

Hidden diversity within the Prussian carp and designation of a neotype for *Carassius gibelio* (Teleostei: Cyprinidae)

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A phylogeny of the genus *Carassius* using the mitochondrial cytochrome *b* gene supported the monophyly and distinctness of the species *C. carassius*, *C. auratus*, *C. langsdorfii* and *C. cuvieri*. In contrast, the samples of *C. gibelio* did not form a monophyletic lineage, but separated into two clades, suggesting the inclusion of two species under the name *C. gibelio*. In order to clarify the identity of *C. gibelio*, a neotype is designated and briefly described.

Introduction

The cyprinid genus *Carassius* is widespread across Europe and North and East Asia. At least five species are considered as valid: *C. carassius* (Linnaeus, 1758) in most of Europe and western Siberia (Kottelat & Freyhof, 2007), *C. langsdorfii* (Temminck & Schlegel, 1846) and *C. cuvieri* (Temminck & Schlegel, 1846) in Japan (Hosoya, 2002; Yamamoto et al., 2010), *C. auratus* (Linnaeus, 1758) in mainland East Asia (Rylková et al., 2010) and *C. gibelio* (Bloch, 1782) in Europe, Siberia and Northeast Asia (Berg, 1949; Kottelat & Freyhof, 2007; Szczerbowski, in Bănărescu & Paepke, 2002). Some authors recognise additionally the species *C. grandoculis* and *C. buergeri* from Japan (Kawanabe & Mizuno, 1989; Suzuki et al., 2005). *Carassius argenteophthalmus* Nguyen & Ngo, 2001 from Northern Vietnam is too poorly described to comments on its identity or validity.

Due to the high morphological similarity between species of *Carassius* and the intraspecific variability of morphological characters (Hensel, 1971; Lusk & Baruš, 1978; Vasileva, 1990; Vasileva & Vasilev, 2000) the definition of species is not always sure, especially in the case of the most widespread species *C. gibelio* and *C. auratus*. In the case of *C. gibelio*, the situation is more complicated by the simultaneous occurrence of diploid ($2n=100$) and triploid ($2n\approx 150$) individuals in many populations (Halačka et al., 2003; Lusková et al., 2004; Abramenko et al., 2004; Mezherin & Lisetskii, 2004; Apalikova et al., 2008). The triploid individuals are usually females that reproduce asexually by gynogenesis and represent clonal lineages (Golovinskaya et al., 1965; Peňáz et al., 1979; Gui & Zhou, 2010), but triploid males have been reported also (Halačka et al., 2003; Abramenko et al., 2004). Kottelat & Freyhof (2007) pointed out that the conspecificity of

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populations with different mode of reproduction or different ploidy level remains to be demonstrated. During the last years, genetic markers have been established that allows differentiation between species. Such genetic analyses have recently shown that *C. auratus* represents a monophyletic lineage that is distinct from *C. gibelio* (Rylková et al., 2010). A number of local lineages within *C. langsdorfii* in Japan, uncovered first by morphological analyses (Hosoya, 2002), later accompanied by genetic data (Takada et al., 2010; Yamamoto, 2010); and genetic data have revealed the existence of at least two lineages among the *C. langsdorfii* that have been introduced to Europe (Kalous et al., 2007; Takada et al., 2010; Tsipas et al., 2009).

While the diversity of *Carassius* in Japan has been objected in several studies (Murakami et al., 2001; Iguchi et al., 2003; Yamamoto et al., 2010; Takada et al., 2010), most *Carassius* in mainland Eurasia are still referred to as *C. gibelio*, despite the fact that the monophyly of these populations has never been confirmed. Kottelat (1997, 2006) pointed out that the basal problem is the poor definition of *C. gibelio*, this means the identity of the species that was described by Bloch (1782) from a European population (in 'Schlesien') under the name *Cyprinus gibelio*.

In the present note we report the presence of two independent lineages within *C. gibelio* as revealed by an analysis of mitochondrial cytochrome *b* sequences. In order to clarify the taxonomic status of *C. gibelio*, a neotype is designated and briefly described.

Material and methods

Phylogenetic analyses. Thirty-four specimens of *Carassius* from European and Asian countries were included in the analysis. In addition to our original samples the dataset contains four sequences coming from previous studies and four sequences obtained from GenBank. As outgroup we have used sequence of common carp, *Cyprinus* sp. Detailed information about used material is listed in Table 1.

Genomic DNA was isolated from ethanol preserved or fresh tissue using DNeasy Blood and Tissue Kit (Qiagen GmbH, Hilden, Germany) according to manufacturer's instructions. The mitochondrial cytochrome *b* gene was amplified using primers Glu L. Ca14337-14359: GAA GAA

CCACCGTTGTTA TTC AA and Thr H. Ca15568-15548: ACC TCC RAT CTY CGG ATT ACA (Šlechtová et al., 2006). PCR amplification was performed in 50 µl reaction volumes containing 15.5 µl Combi ppp Master Mix (Top-Bio s.r.o., Praha, Czech Republic), 3 µl of each primer and template DNA. The PCR profile started with 10 min period of initial denaturation at 95 °C, followed by 34 cycles each consisting of denaturation step at 94 °C for 30 s, annealing step at 54 °C for 30 s and elongation step at 72 °C for 1 min. PCR was terminated by final elongation step at 72 °C for 10 min. PCR was carried out on MJ Mini thermocycler (Bio-Rad Laboratories, Hercules, CA, USA). PCR products were purified and sequenced by Macrogen Inc., Seoul, Korea.

The raw chromatograms were manually assembled and checked by eye for potential mistakes using computer software BioEdit 5.0.9 (Hall, 1999); the same program was used to align sequences using the ClustalW algorithm.

The phylogenetic relationships were estimated from aligned sequences using the method of maximum parsimony (MP) performed in PAUP* version 4.0b10 (Swofford, 2000) and Bayesian analysis (BAY) using the program MrBayes ver. 3.0 (Huelsenbeck & Ronquist, 2001) as described in Šlechtová et al. (2004).

Morphological data. Measurements and counts were done according to Kottelat & Freyhof (2007) using digital callipers. All measurements were recorded to the nearest 0.1 mm. Number of fin rays, vertebrae and ribs were taken from digital high-resolution radiographs, using a digital X-ray device Faxitron LX-60.

Ploidy determination. The ploidy level was determined using the measurements of erythrocyte nuclei area by computer-assisted image analyses as was proposed by Flajšhans (1997). Prior to any handling, the fish were anaesthetized with 0.6 ml · l⁻¹ 2-phenoxyethanol (Merck KGaA). Blood was taken from the heart by a heparinised syringe; blood smears were prepared on clean microscope slides one for each specimen and fixed by few drops of 90 % ethanol. Slides were stained in a 20 % Giemsa-Romanowski solution for 10 minutes. Computer-assisted image analysis was carried out using a system that was composed from a microscope Nikon Eclipse 600 with immersion objective 100×, an analogue video camera Hitachi HVC 20 and the software L.U.C.I.A

ver. 4.71. The mean area of nuclei was calculated from 247 erythrocytes of the neotype and 220 and 200 erythrocytes of diploid and triploid reference specimens, respectively. The triploid reference specimen (156 chromosomes) originated from Řehačka backwater (alluvium of Elbe River), Central Bohemia, Czech Republic 50°10'39"N 14°48'27"E and it is deposited in the National Museum Prague (NMP P6V140484). The diploid reference specimen is a goldfish (*Carassius auratus*) var. Oranda (100 chromosomes) from petshop in Prague. Karyotype analyses of reference specimens were performed according to Ráb & Roth (1988). The calculated nuclei areas were compared by t-test in programme STATISTICA ver. 9.

Results

The final matrix of the cytochrome b sequences consisted of 931 characters containing 222 variable characters with 157 parsimony informative sites. Both employed methods have recovered trees of very similar topologies with high statistical supports and sorted sequences into six well-supported lineages (Fig. 1).

The neotype specimen had a mean erythrocyte nuclei area of 15.32 μm^2 (SD 2.06 μm^2), which is significantly smaller (t-test, $p < 0.01$) than erythrocyte nuclei of the triploid reference specimen (21.58 μm^2 , SD 3.12 μm^2) and corresponds to the values of the diploid reference specimens

Table 1. Material used for the genetic analyses. Sources: a, Takada et al. (2010); b, Rylková et al. (2010); c, Kalous et al. (2007); n, GenBank database – unpublished; *, present study.

taxon	Acc. No.	source	origin
<i>Carassius auratus</i>	EU663599	b	Wuhan, Yangtze River, China
	GU991398	*	Gyeongju, Miho-cheon River, Korea
	EU663597	b	Nanking, Yangtze River, China
	GU991392	*	Nanking, Yangtze River, China
	GU991386	*	Ochrid Lake, Albania
	GU991390	*	Ishem River, Albania
	GU991395	*	Prespa Lake, Greece
	EU663574	b	pet shop, Czech Republic
	GU991391	*	Shuchinsk, Ishim River drainage Kazakhstan
<i>C. gibelio</i> I	HM000009	*	Czerskie Rumunki, Vistula River, Poland
	HM000020	*	Haaslava, Estonia
	GU170378	n	Volga River, Russia
	FJ822041	n	Hanka (Khanka) Lake, Primorye, Russia
	FJ478019	n	Lake near Dalnegorsk, Primorye, Russia
	AB368700	a	Amur River, Russia
	HM000008	*	Oltenița, Danube River, Romania
	HM008678	*	Varna, Bulgaria
	JN402305	*	neotype; Český Těšín, Olza River, Czech Republic
	HM008684	*	Byur Lake, Amur drainage, Mongolia
HM008685	*	Byur Lake, Amur drainage, Mongolia	
<i>C. gibelio</i> II	DQ868924	*	Uvs Lake, Mongolia
	DQ868925	*	Uvs Lake, Mongolia
	DQ868926	*	Uvs Lake, Mongolia
	HM008690	*	Bulgan, Selenga River, Mongolia
<i>C. langsdorfi</i>	AB368690	a	Honshu, Biwa Lake, Japan
	DQ399920	c	Hokkaido, Abashiri Lake, Japan
	AB368677	a	Taktsu, Honshu, Japan
	AB368678	a	Iki Island, Japan
	AB368680	a	Okinawa Island, Japan
<i>C. cuvieri</i>	AB045144	n	unknown
	JN402304	*	Honshu, Lake Mikatako, Japan
<i>C. carassius</i>	DQ399917	c	Plön, Germany
	GU991400	*	Calverton, Great Britain
	DQ399938	*	Milevsko, Elbe drainage, Czech Republic
<i>Cyprinus</i> sp.	HM008692	*	Mekong River, Thailand

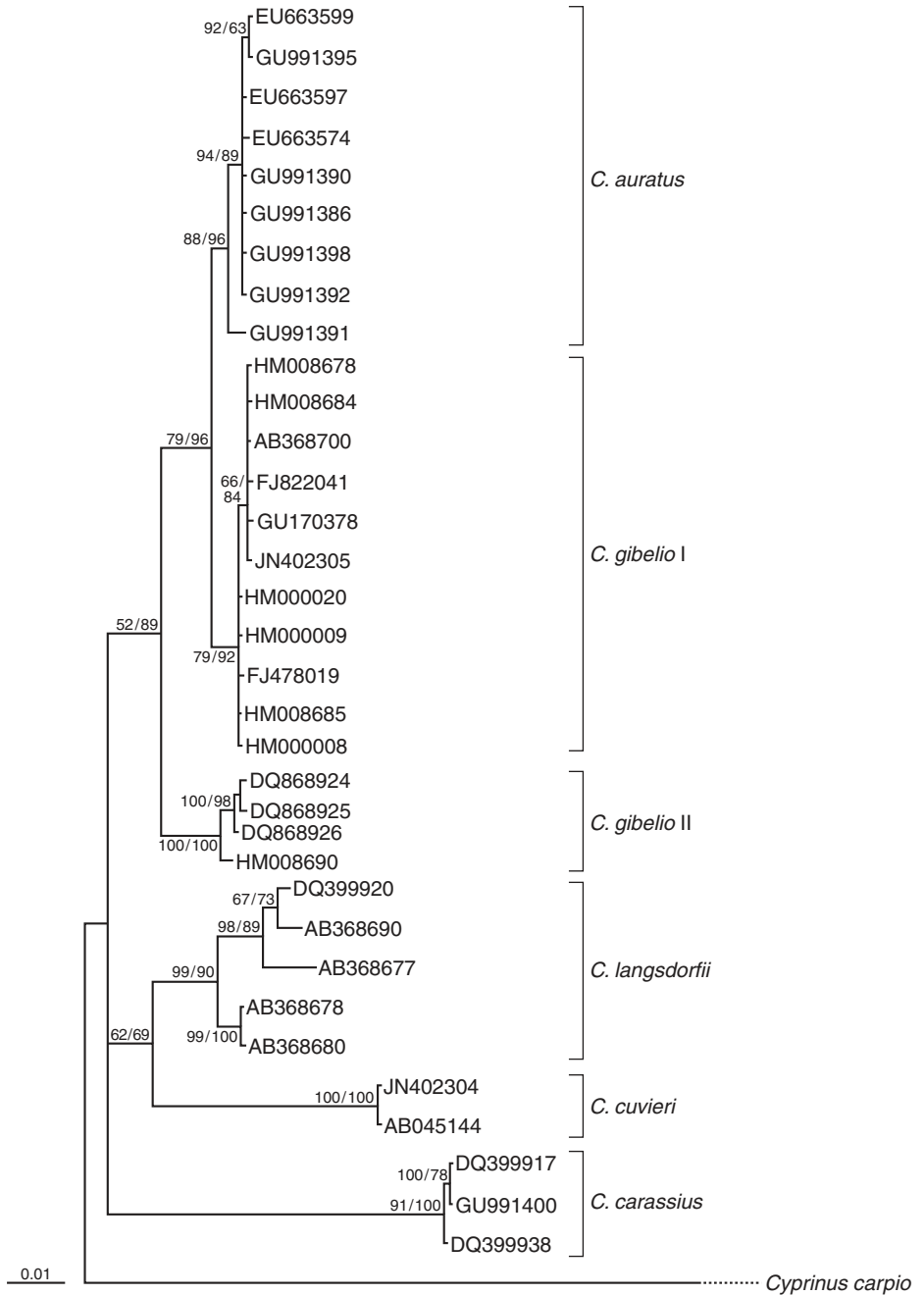


Fig 1. Reconstructed phylogeny of the cytochrome *b* sequences of *Carassius* samples included in present study. Numbers at nodes represent statistical supports for BAY and MP analyses respectively.

(14.82 μm^2 , SD 1.8 μm^2). The respective values are in agreement with published results confirmed by karyological analyses where triploids and

diploids of *C. gibelio* are characterized by nuclei area of $14.03 \pm 1.46 \mu\text{m}^2$ and $20.71 \pm 1.76 \mu\text{m}^2$ respectively (Kalous & Petrtýl, 2004).



Fig. 2. *Carassius gibelio*, ZMB 33979, neotype, male, 127.4 mm SL; Czech Republic: Silesia: Odra River system.

Discussion

Our data show six separate genetic lineages within the genus *Carassius*. Four of these correspond to the species *C. carassius*, *C. auratus*, *C. langsdorfii* and *C. cuvieri*, respectively. In contrast, the samples of *C. gibelio* do not form a monophyletic lineage, but separate into two clades. One of these clades contains all samples of *C. gibelio* from western Mongolia, while the other clade collects samples of *C. gibelio* from Europe, Russian Federation, eastern Mongolia and China. The two clades do not have sister-relation to each other; instead, the Europe-China clade is more closely related to *C. auratus* than to the second clade of *C. gibelio*.

The present results suggest that the genus *Carassius* contains a higher diversity than formerly known, and that at least two species are included within what is presently considered as *C. gibelio*. Prussian carps from western Mongolia have been morphologically investigated by Peñáz & Dulmaa (1987) and identified as *C. gibelio*. Kottelat (2006) pointed on the problems of identification and nomenclature of Mongolian *Carassius* that come from the missing definition of *C. gibelio*. For further studies on the taxonomy of Prussian carps it is important to define which of the two species represents *C. gibelio*.

Cyprinus gibelio was described by Bloch (1782) and was stated to occur in 'Churmark, Pommern,

Schlesien und Preussen', corresponding nowadays to most of eastern Germany, Poland and a part of north-eastern Czech Republic. Bloch did not explicitly designate a holotype; consequently all specimens included by Bloch are syntypes (ICZN, 1999, arts. 72.2, 73.4). In the part of Bloch's collection still present in Museum für Naturkunde (ZMB) in Berlin, a single lot is catalogued as *C. gibelio*, but nowadays this lot contains a specimen of *C. carassius* (see Paepke, 1999). Paepke (1999) demonstrated that the original syntype of *C. gibelio* has been replaced by a specimen of *C. carassius* during former investigations. No other potential types have ever been reported. During a research visit in ZMB in 2001, LK together with the staff of the museum searched the collection again for potential syntypes of *C. gibelio* but failed to find any. We therefore conclude that all type specimens of *C. gibelio* are lost. Since the present study indicates that more than one species is hidden under the name *C. gibelio*, a neotype designation is needed to fix the name *C. gibelio* to one of the identified species. We here designate specimen ZMB 33979 as neotype of *C. gibelio* (Fig. 2). The specimen originated from an alluvium area close to Český Těšín in the historical area of Silesia [Schlesien], one of the areas mentioned in the original description of *C. gibelio*. In accordance with ICZN (1999) art. 73.3.6, the neotype therefore comes from a locality that is part of the type locality. The neotype

corresponds in all investigated morphologic characters to the description of *C. gibelio* as given by Bloch (1782). In order to avoid taxonomical problems that might arise from the high percentage of polyploid specimens of gynogenetically reproducing lineages, we selected an adult male as neotype as indicated by the presence of spawning tubercles. According to the size of its erythrocyte nuclei, the neotype specimen is diploid ($2n=100$). A description of the neotype is given below.

Table 2. Morphometric and meristic data of neotype of *Carassius gibelio*, ZMB 33979.

	in mm in % SL	
Total length	163.0	127.9
Standard length	127.4	100.0
Lateral head length	37.5	29.4
Predorsal length	63.6	49.9
Prepectoral length	36.1	28.3
Prepelvic length	61.8	48.5
Preanus length	91.7	72.0
Preanal length	94.6	74.3
Snout length	11.8	9.3
Horizontal eye diameter	7.3	5.7
Interorbital width	15.6	12.2
Head depth at eye	23.2	18.2
Head depth at nape	33.2	26.1
Body depth at dorsal-fin origin	51.1	40.1
Body depth at anal-fin origin	36.3	28.5
Depth of caudal peduncle	19.5	15.3
Length of caudal peduncle	21.0	16.5
Head width at eye	19.2	15.1
Maximum head width	25.4	19.9
Body width at dorsal-fin origin	26.0	20.4
Body width at anal-fin origin	17.2	13.5
Height of dorsal fin	24.3	19.1
Length of upper caudal-fin lobe	34.7	27.2
Length of middle caudal-fin ray	20.6	16.2
Length of lower caudal-fin lobe	36.8	28.9
Height of anal fin	20.9	16.4
Length of pelvic fin	26.0	20.4
Length of pectoral fin	25.9	20.3
Number of pores in lateral line	28	
Number of scales along lateral line	26+2	
Number of transverse scales between lateral line and origin of dorsal fin	1/26	
Number of transverse scales below lateral line in front of pelvic fin	8	
Number of rows of scales around caudal peduncle	16	
Number of branched dorsal-fin rays	18 1/2	
Number of branched caudal-fin rays	9+8	
Number of branched anal-fin rays	5 1/2	
Number of pelvic-fin rays	9	
Number of pectoral-fin rays	18	

The neotype was analysed genetically for the present study and is part of the Europe-China clade of *C. gibelio*. Consequently, the name *C. gibelio* can be used for this lineage, while a different name has to be given for the Mongolian clade. We do not have sufficient material for a detailed morphological analysis of the Mongolian clade; but preliminary data (LK, unpubl.) suggest that this lineage does not correspond to any of the already available species names.

Our genetic analyses grouped most specimens from Albania and Greece (river Ischem and Lakes Prespa and Ochrid) to *C. auratus*, although only *C. gibelio* was formerly reported from the Balkan region (e.g. Perdikaris et al. 2012). It is possible that feral populations of *C. auratus* have been wrongly identified as *C. gibelio*. Further investigation is needed to identify the species occurring in the Balkan region.

Carassius gibelio

(Fig. 2)

Neotype. ZMB 33979, male, 127.4 mm SL, Czech Republic: pond in alluvium area of Olza River (tributary of Odra River) at Český Těšín; 49°47' 11" N 18°35'24" E; Lukáš Choleva, 5 May 2011.

Description. Morphometric and meristic data of neotype are shown in Table 2. Head and body laterally compressed; body high, greatest depth before dorsal-fin origin. Dorsal fin with 5 unbranched and 18 1/2 branched rays; last unbranched ray with 10 spines along posterior edge on distal 60 % of length. Caudal fin with 9+8 branched rays, lower lobe slightly longer than upper. Pelvic fin with 9 rays, not reaching anus, which is located directly before anal-fin origin; origin under last unbranched dorsal-fin ray. Pectoral fin with 18 rays, reaching backwards to base of pelvic fin. Anal fin with 3 unbranched and 5 1/2 branched rays, origin under branched dorsal-fin ray 13; last unbranched ray with 10 spines along posterior edge on distal 60 % of length; base reaching posteriorly beyond base of dorsal fin. Anal fin not reaching caudal fin. Breeding tubercles under eye and on opercle, along dorsal surface of first pectoral-fin ray and on median branched pelvic-fin rays at about 60 % of their length. Number, size and arrangement of tubercles of left and right sides different. First gill arch with 47 gill rakers. Total number of vertebrae 28, 14 ribs on each side.

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