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Genetic diversity in the genus *Carassius* (Teleostei: Cyprinidae) in the Czech Republic

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Abstract. Molecular markers have recently been used to change the taxonomical status of many species of fish. Fishes of the genus *Carassius* (Nilsson, 1832) were subjected to phylogenetic analyses when it was recognized that differences in morphology could not be used for identifying species. Four species of *Carassius* are known to occur in waters of the Czech Republic: *Carassius carassius* (Linnaeus, 1758), *C. gibelio* (Bloch, 1782), *C. auratus* (Linnaeus, 1758) and *C. langsdorfii* (Temminck et Schlegel, 1842). In order to obtain an insight into the genetic structure of Czech populations, the mitochondrial gene cytochrome *b* of sixty five samples of *Carassius* collected from different localities were analyzed. This analysis identified four of the currently recognized species as mitochondrial lineages of which that at least lineage of *C. langsdorfii* can be further divided. Moreover, a fifth lineage not corresponding to any of the described species was also revealed. Thus, the genetic diversity of the genus *Carassius* in the Czech Republic is greater than previously thought.

Key words. Ichthyology, phylogenetics, molecular analysis, *Carassius carassius*, *Carassius gibelio*, *Carassius auratus*, *Carassius langsdorfii*, cytochrome *b*, Czech Republic.

INTRODUCTION

The ichthyofauna of the Czech Republic is not one of the richest in the world as it includes only 105 species (Froese & Pauly 2012). There was considerable attention concentrated to ichthyologic research in the past century and also present time (Baruš & Oliva 1995, Šanda 2006, Szczerbowski 2002). However, we still know very little about the overall diversity of fish, especially in those genera in which the species are morphologically similar although distinct in many other characters (Janko et al. 2007, Choleva et al. 2008, Mendel et al. 2008). For example, the diversity of species in the genus *Carassius* has been the subject of many scientific discussions (Hensel 1971, Kottelat 1997, Kalous & Bohlen 2002, Lusková et al. 2004) and the phylogenetics and corresponding taxonomical structure is continuously changing. Recently it was recognized that there were four species of the genus *Carassius* in waters of the Czech Republic. Native crucian carp *Carassius carassius* (Linnaeus, 1758) was abundant in the past but now natural populations are rare and decreasing (Lusk et al. 2011). Spreading of the more resistant polyploid complex of *C. gibelio* and disappearing of suitable habitats is considered as main reasons of this threat (Kottelat & Freyhof 2007). The diploid form of Prussian carp (*C. gibelio* Bloch, 1782), which was described from Silesia and other regions of north central Europe by Bloch, is native in the north–east tip of the Czech Republic (Kalous et al. 2012). The non-native polyploid complex represented mainly by gynogenetic populations with a significant female bias invaded Czech waters in the late seventies via the River Danube (Holčík & Žitňan 1978, Lusk et al. 1980, Halačka et al. 2003). Recently Japanese Ginbuna (*C. langsdorfii* Temminck et Schlegel, 1842) was recorded at one locality in eastern (Kalous et al. 2007) and another in southern (Rylková et al. in press) Bohemia, but their

Table 1. List of haplotypes found in samples of *Carassius* collected in the Czech Republic and recorded in following sources of data: a - Rylková et al. (2010); b – Kalous et al. (2007); c – Papoušek et al. (2008); d – Rylková et al. (2013). Abbreviation: CZ – Czech Republic

taxon	haplotype	origin	GenBank acc. No.	reference
Carassius auratus	*Ca1*	fish farm, Vodňany, CZ	GU991385	d
	Ca2	fish farm, Vodňany, CZ	DQ868897	a
		fish farm, Vodňany, CZ	DQ868898	d
		fish farm, Vodňany, CZ	DQ399924, DQ399925	b
		pet shop, Mělník, CZ	DQ399923	b
		pet shop, Prague, CZ	EU663576, EU663578, EU663579, EU663580, EU663581, EU663582, EU663583, EU663585, EU663589, EU663590	a
		pet shop, Prague, CZ	DQ868927	b
	Ca3	pet shop, Prague, CZ	EU663577	a
	Ca4	pet shop, Prague, CZ	EU663575	a
	Ca5	pet shop, Prague, CZ	EU663584, EU663586, EU663587 EU663588	a
	Ca6	pet shop, Prague, CZ	EU663574	a
Carassius gibelio	*Cg1*	Dolní Benešov, CZ	DQ399927	b
		fish farm, Hluboká nad Vltavou, CZ	DQ399933, DQ399934, DQ399935	b
	Cg2	Stráž nad Nisou, CZ	DQ399928	b
	Cg3	Chrudimka River, CZ	DQ399929, DQ399931	b
		Strkov, Lužnice River, CZ	DQ399936, DQ399937	b
		Dyje River, CZ	DQ399939, DQ399940	b
		Dyje River, CZ	HM000035, HM000030	d
		pool Řehačka, CZ	JN546034, JN546040, JN546041, JN546043, JN546055, JN546056, JN546057, JN546058	d
		Berounka River, CZ	JN546044, JN546045	d
	Cg4	Berounka River, CZ	JN546046	d
	Cg5	Dolní Benešov, CZ	DQ399926	b
	Cg6	Dyje River, CZ	DQ868905, HM000031, HM000032, HM000033, HM000034	d
	Cg7	Olza River, CZ	JN402305	d
		pool Řehačka, CZ	JN546042	d
Carassius sp. "M"	*Cm1*	Dyje and Morava River confluence, CZ	FJ169954	c
Carassius langsdorfii	*Clan1*	Chrudimka River, CZ	DQ399930, DQ399932	b
	Clan2	pool at Litvínovice, CZ	JN412529, JN412530	d
Carassius carassius	*Cc1*	fish farm Višňová, CZ	JN412549, JN412550	d
	Cc2	Milevsko, CZ	GU991399	d
	Cc3	Milevsko, CZ	DQ399938	b
	Cc4	pool near Řehačka settlement, CZ	KC238569	this study
Cyprinus carpio		Mekong River, Thailand	HM008692	d

origin is unknown although imports of koi carp and its non-intentional release are suggested by Kalous et al. (2007). Goldfish (*C. auratus* Linnaeus, 1758) are not native in the Czech Republic (Baruš & Oliva 1995) and its occasional occurrence is associated with the ornamental trade and accidental or intentional releases by the public (Kottelat 1997). With the exception of *C. carassius*, fishes of the genus *Carassius* do not have clear unique morphological characters that can be used to identify them. Therefore, molecular markers were employed to reveal the diversity of fishes within the genus *Carassius* in the Czech Republic.

MATERIAL AND METHODS

Material

Altogether sixty five samples of *Carassius* fishes originating from the Czech Republic (Fig. 1) were included in this study. Sixty three samples came from our previous studies, one is original to this study and one was obtained from GenBank database. As an outgroup we used a sequence of Common carp (*Cyprinus carpio* Linnaeus, 1758) obtained from GenBank acc. No. HM008692. Detailed information on the material sampled is listed in Table 1.

DNA isolation, PCR amplification and Sequencing

Genomic DNA was isolated from ethanol preserved tissue using DNeasy Blood and Tissue Kit (Qiagen) according to manufacturer's protocol. The mitochondrial gene cytochrome b was amplified using forward primer Kai_F5'-GAA GAA CCA CCG TTG TTA TTC-3' and reverse primer Kai_R5'-ACC TCC RAY CTY CGG ATT ACA-3' (Šlechtová et al. 2006). Polymerase chain reaction (PCR) consisted of 3µl template DNA, 3 µl of each primer, 15.5 µl of Combi ppp Master Mix (Top-bio) and ddH$_2$O up to 50 µl of the reaction mix. The profile of the PCR, carried out on an MJ Mini™ thermocycler (Bio-Rad) is described in Rylková et al. (2010).

PCR products were purified and sequenced from both ends to obtain a complete sequence of the gene. Purification and sequencing were performed by Macrogen Inc., Korea.

Reconstruction of Phylogeny

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

The phylogenetic relationships were estimated using the methods of maximum parsimony (MP) in PAUP* version 4.0b10 (Swofford 2000) and Bayesian analyses (BAY) using the program MrBayes ver. 3.0 (Huelsenbeck & Ronquist 2001).

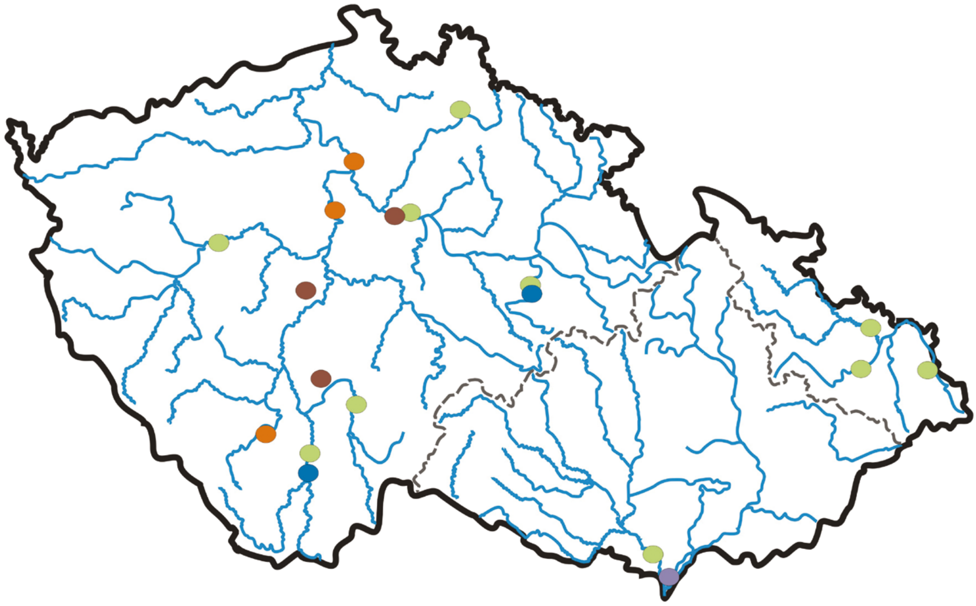

Fig. 1. Map of the Czech Republic showing the main rivers and sites where samples of *Carassius* were collected. The occurrence of five species of *Carassius* was confirmed using molecular analysis: Ca – *Carassius auratus* (orange circle), Cg – *C. gibelio* (green circle), Clan – *C. langsdorfii* (blue circle), Cc – *C. carassius* (brownish circle), C "M" – *Carassius* sp. (purple circle). For details of occurrence see Table 1.

RESULTS

The final matrix of the cytochrome b sequences consisted of 1112 basepairs containing 250 variable characters with 143 parsimony informative sites. Altogether, the analyzed specimens of *Carassius* revealed 20 haplotypes. Both of the methods used recovered trees of very similar topologies with high statistical supports and sorted sequences into five well-supported lineages (Fig. 2).

Four lineages correspond to the species *Carassius auratus*, *C. gibelio*, *C. langsdorfii* and *C. carassius*. The fifth lineage labelled *Carassius* "*M*" does not correspond to any described species

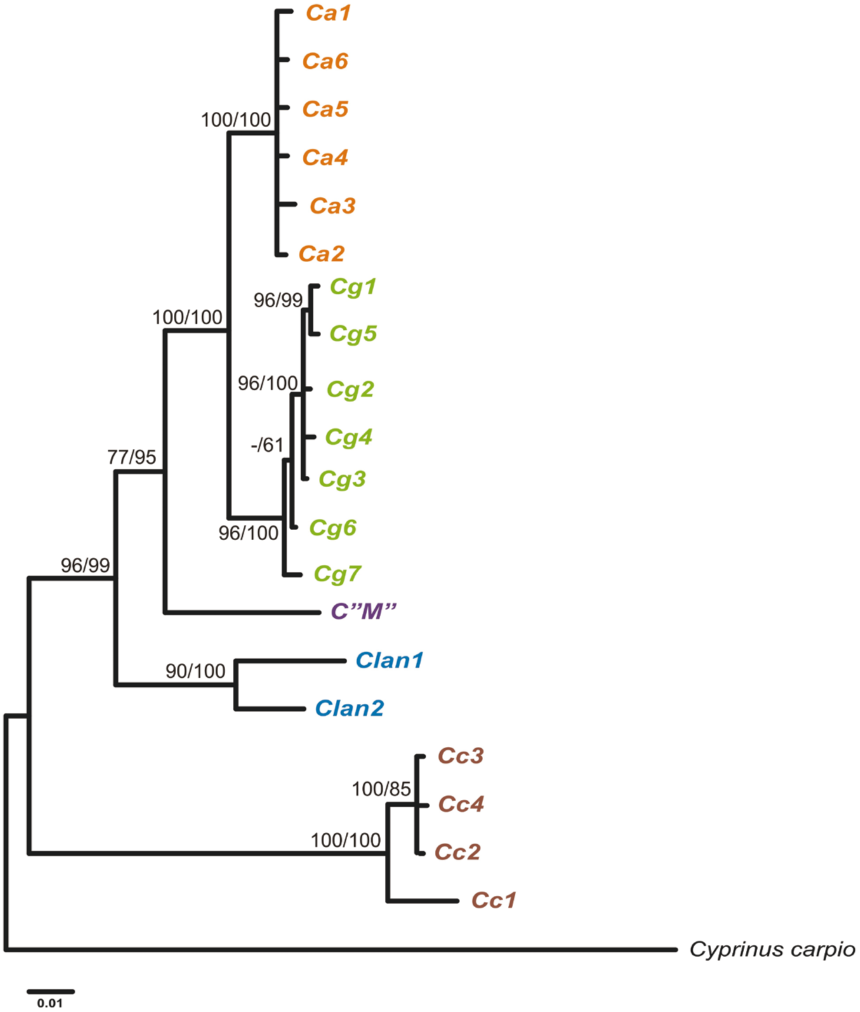

Fig. 2. Phylogenetic tree. Reconstructed phylogeny based on cytochrome *b* sequences of *Carassius*. The numbers at the nodes represent statistical supports for BAY and MP analyses, respectively. The bootstrap supports below 50 and Bayesian posterior probabilities below 0.75 are not shown or are represented by a dash.

and forms a cluster of its own. There are six haplotypes in the *C. auratus* lineage, seven in that of *C. gibelio*, two in *C. langsdorfii*, one in *Carassius* "*M*", and four in *C. carassius*.

DISCUSSION

The diversity within the genus *Carassius* is higher than expected based on the available information.

All specimens of *C. auratus* originated either from pet shops or fish farms. We have not identified any goldfish from a natural environment. This confirms the supposition that the climate is probably too cold to allow it to survive and establish feral populations in the Czech Republic. Ginbuna (*C. langsdorfii*) was found at two separate localities. This is not surprising since recently many findings have been reported in Europe (e.g. Kalous et al. 2007, Tsipas et al. 2009, Kalous et al. 2013). The difference in the genetics of the specimens from the two localities is significant (3.69%). Phylogenetic analyses of Takada et al. (2010) reveal high intra-specific diversity suggesting that several distant taxa are recently hidden under the name *C. langsdorfii*. Comparison of the results of both analyses shows that in the Czech Republic there are two separate taxa, which are likely to have different origins. Haplotypes and geographical distribution of *C. gibelio* do not match. Genetic structure is random reflecting its extraordinary ability of spreading and numerous artificial translocations. Only haplotype Cg5 is unique to the River Dyje. Although there should be a native diploid population of *C. gibelio* in the Odra Basin it is likely interbred with genotypes from another region and is most likely incorporated in the invasive diploid-polyploid complex of *C. gibelio*. The identification of the native population is now very complicated. The lineage of *C. carassius* is also interesting. Among the five specimens analyzed there were four haplotypes with an internal cluster variability of 2.16%. Similar results but on a larger geographic scale is recorded in other studies (Rylková et al. 2013, Chen et al. 2012). This high value suggests that the genetic diversity is worthy of further study. The crucian carp (*C. carassius*) is currently disappearing species and its status is poorly reflected in environmental regulation. Although in *The Red List of Lampreys and Fishes of the Czech Republic* its status is at level critically endangered (CE) with rapid decreasing of population over the last fifty years (Lusk et al. 2011), according to the law *C. carassius* does not belong among protected species in the Czech Republic (Act No 114/1992). The fifth lineage *C.* "*M*" could not be assigned to any known species. The taxonomic status of this lineage is discussed in Kalous et al. (2012) who indicated that it may be a new species.

The genetic diversity within the genus *Carassius* is further complicated by the formation of diploid – polyploid complexes that may composed of several mitochondrial lineages (Rylková et al. 2013). Moreover, there are inter-specific hybrids among *C. carassius* and *C. gibelio* and *C. auratus* (Hänfling 2005, Papoušek et al. 2008), which pose further threats to *C. carassius* and native biotype of *C. gibelio*.

CONCLUSION

In the Czech Republic five mitochondrial lineages, which represent either independent species (*Carassius carassius*, *C. gibelio*, *C. auratus*, *C. langsdorfii*) or are incorporated in diploid-polyploid complexes of *C. gibelio* and *C. langsdorfii* are recognized. It is also likely that there are inter-specific hybrids between *C. auratus*, *C. carassius* and *C. gibelio*.

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