

NEW FINDING OF NON-INDIGENOUS JAPANESE CYPRINID FISH IN THE CZECH REPUBLIC*

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Ginbuna *Carassius langsdorfii* endemic species of Japanese archipelago have been found in European waters. The origin of European records and the environmental impact remain unclear. The present paper reports on the population of this species discovered in a small natural pool in South Bohemia. Genetic markers were employed since morphological characters failed in species identification. Although the population was clustered in the mitochondrial lineage of *C. langsdorfii*, genetic distance and morphological difference were found largely significant when comparing to other ginbuna individuals found in Europe. The phylogenetic position is further discussed.

Carassius; introduction; South Bohemia; cytochrome *b*; phylogeny

INTRODUCTION

Although the Czech waters are not much rich for freshwater ichthyofauna in worldwide context, they encompass a high percentage of non-native fishes that are represented by 41 species (Lusk et al., 2010). The reasons for introductions in the last century were mainly aquacultural as well as experimental to fill empty niches in semi-natural environment of Czech rivers and streams. Some introductions were also promoted to satisfy the enlarging community of recreational fishermen as it was for example in the case of *Oncorhynchus mykiss* (Lusk et al., 2010).

One of the globally successful genera that undergone naturalization in many places of the world is the genus *Carassius* (Brumley, 1996; Dyer, 2000; Elvira, 2001; Copp et al., 2005; Musil et al., 2010).

Four species of the genus *Carassius* (*sensu* Rylková et al., 2010) are recognized in the Czech water bodies: Crucian carp *C. carassius* (L.), invasive gynogenetic biotype of Prussian carp *C. gibelio* (Bloch, 1782), domesticated or feral forms of introduced Goldfish *C. auratus* (L.), and recently recorded ginbuna *C. langsdorfii* (Temminck, Schlegel, 1842). The last three mentioned species, namely *C. gibelio*, *C. auratus*, and *C. langsdorfii*, are included in so called *Carassius auratus* complex, mainly because of their morphological similarity, hybridization, and

not completely solved taxonomical status (Takada et al., 2010).

C. gibelio and *C. langsdorfii* are characteristic for their capability of clonal reproduction via gynogenesis, occurrence of all female populations consisting of polyploid individuals which sexually parasite on other cyprinid fishes (e.g. Gui, Zhou, 2010). These features, like all female population and clonality, allow rapid invasive spreading into new areas and led to the consideration of fishes from *C. auratus* complex as of animals with high environmental impact (Savini et al., 2010).

The ginbuna originated from Japanese archipelago where it is considered a common species (Hosoya, 2000) but its appearance in Europe was evaluated as accidental and rare (Kalous et al., 2007). The same authors tentatively attributed its introduction to Europe as results of the Koi carps imports. However, after Kalous et al. (2007), another finding from the Elbe River basin was recorded in Greek lakes (Tsipas et al., 2009; Takada et al., 2010). Latest screening of Kalous et al. (2013) revealed the presence of *C. langsdorfii* at other five European localities. Beside the south European countries (Italy, Bosnia and Herzegovina, and Greece) it was found also in northern Germany. Another finding of *C. langsdorfii* from South Bohemia with distinct morphology and phylogenetic position is presented herein.

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MATERIAL AND METHODS

Samples

In 2007 several fish of atypical appearance (Fig. 1) were caught in a small natural pool near Litvínovice (South Bohemia; 48°57'34.96"N, 14°27'13.70"E). These individuals were brown-green at dorsal side and dark yellow up to orange at ventral side. All fins were reddish-brown in colour. Upper edge of the dorsal fin was slightly concave up to almost straight. Number of scales in lateral line 30–33; number of scales both above and below lateral line 6–7; number of dorsal fin rays III 17 ($n = 6$). With respect to this, morphological characters were not typical of any *Carassius* species occurring in European waters. Since morphological characters are known to be not much reliable in determination of species within the genus *Carassius* (Hensel, 1971; Vasileva, 1990), molecular markers were employed to identify the species affiliation.

Altogether 41 specimens of *Carassius* were included into the analysis. As outgroup, the sequence of common carp *Cyprinus carpio* was used. Detailed information on the samples origin and GenBank Accession Nos. are 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, Valencia, USA) according to manufacturer's instructions. Mitochondrial gene cytochrome *b* was amplified using the forward primer Kai_F (5' GAA GAA CCA CCG TTG TTA TTC 3') and reverse primer Kai_R (5' TTA GTT TCT TTT CCT CCG CT 3') (Šlechťová et al., 2006). PCR was performed in 50 µl reaction volumes as described in Rylková et al. (2010). The PCR profile (carried out on MJ Mini thermocycler, Bio-Rad, Hercules, USA) started with 10 min period of initial denaturation step at 94°C, followed by 34 cycles, each consisting of denaturation step at 94°C for 30 s, a primer annealing step at 54°C for 30 s, and an elongation step at 72°C for 1 min. PCR was



Fig. 1. Specimen from the Litvínovice pool (photo by J. Okrouhlik)

terminated by final elongation period at 72°C for 10 min. PCR products were purified and sequenced from both (3' and 5') ends of fragments using the same pair of primers as used for double strand PCR amplification. Purification and sequencing were performed by MacroGen Inc., Seoul, Korea.

Molecular data analyses

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 analysis (BAY) using the program MrBayes, version 3.0 (Huelsenbeck, Ronquist, 2001) as described in Rylková et al. (2010).

To estimate the "fine scaled" relationships among *C. langsdorfii* haplotypes, we constructed a haplotype network employing the statistical parsimony (Templeton et al., 1992) implemented in the TCS 1.21 program (Clement et al., 2000). The connection limit was set to 20 mutation steps.

RESULTS AND DISCUSSION

The final matrix of the cytochrome *b* sequences consisted of 1082 basepairs containing 255 variable characters with 159 parsimony informative sites. Both employed methods have recovered trees of very similar topologies with high statistical supports and sorted the sequences into 5 well-supported lineages corresponding to *C. langsdorfii*, *C. auratus*, *C. gibelio*, *C. cuvieri*, and *C. carassius*, respectively (Fig. 2).

There are 21 haplotypes within the clade of *C. langsdorfii* showing high genetic diversity within this taxon. The whole lineage is clearly divided into 3 clusters: cluster *I* (haplotypes 1–12), cluster *II* (haplotypes 13–15), and cluster *III* (haplotypes 16–21). Specimens coming from South Bohemia belong to haplotype *Clan16* nested in cluster *III*.

Haplotype network analysis (Fig. 3) divided the lineage of *C. langsdorfii* into three separate groups corresponding to cluster *I*, *II*, and *III* of the phylogenetic tree.

The presented analysis showed a high phylogenetic diversity within the lineage of *C. langsdorfii*. Specimens coming from South Bohemia are quite distant from those recorded in the upper part of the Elbe Basin (Kalous et al., 2007) what is further accompanied by different values on morphological characters. Origin of the fish from both Czech findings remains unclear, but most probably each population belongs to different introduction events. The South Bohemian population

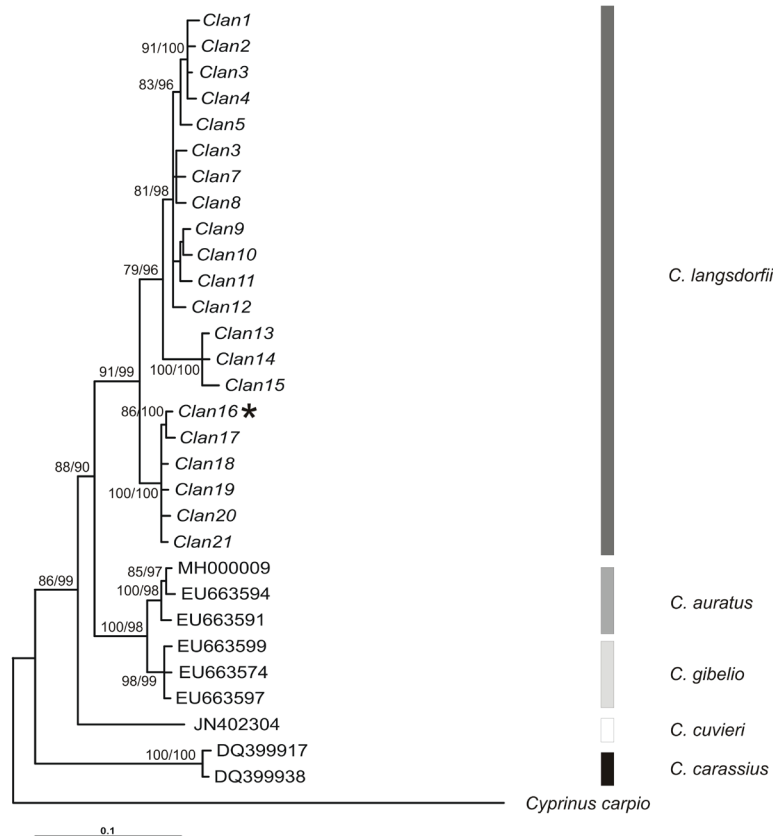


Fig. 2. Reconstructed phylogeny of the cyt b sequences of *Carassius* included into the present study. Numbers at the nodes represent statistical supports for maximum parsimony (MP) and Bayesian analysis (BAY), respectively. *haplotype including the fish from Litvínovice

(*Clan16*) is clustered with samples deriving from Ryukyu Island (*Clan16*, *Clan18-Clan21*) but that from the Chrudimka River (*Clan13*) is linked to samples from Honshu Island (*Clan15*). The population of *C. langsdorfii* from several Greek lakes is also interesting (Tsipaset al., 2009). Part of it shares the same haplotype with the specimens from the Chrudimka River, while the other part (*Clan17*) is very closely related to South Bohemian population. This indicates that both clusters of *C. langsdorfii* are more spread in European waters.

The haplotype analysis sorted the samples of the *C. langsdorfii* lineage into three separate groups. This fact further supports the presumption that *C. langsdorfii* may consist of more taxa. It has already been mentioned by Murakami et al. (2001) and Takada et al. (2010) that several species are probably taxonomically treated under the name *C. langsdorfii*. This fact must be firstly proven and resolved at the place of natural occurrence of these fishes. Having in mind all the above-mentioned information, we recommend to treat the population found in South Bohemia as *Carassius* cf. *langsdorfii* since its taxonomical status seems to be problematic.

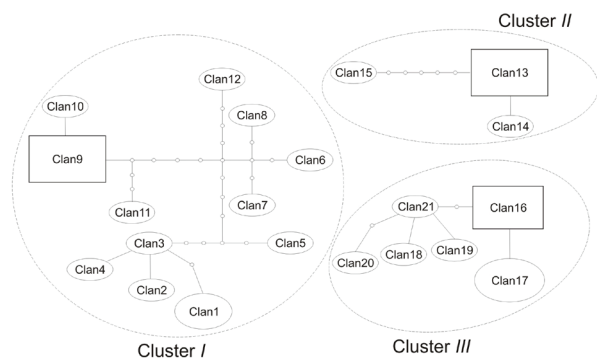


Fig. 3. Unrooted haplotype network based on cyt b sequences of *C. langsdorfii* analyzed. The haplotype numbers refer to numbers in Table 1. and Fig. 2. The oval area is proportional to the haplotype frequencies

The population of gimbuna has shown itself ecologically very strong in a small pool in the inundation area; in fact it was dominant throughout many years of observations (1999–2006). It represented the majority (> 50%) of the fish community; the supplemental species were topmouth gudgeon (*Pseudorasbora parva*) and common tench (*Tinca tinca*). Gimbuna was apparently reproducing itself in the pool as the young-of-the-year fish always dominated. The dominant position of gimbuna was most likely supported by the harsh oxygen conditions during the winter to which the *Carassius* species are known to be more tolerant than the other fish (Blažka, 1958; Blažka et al., 2006). The pool at Litvínovice was flooded by the Vltava River water

Table 1. Material used for the genetical analyses

Species	Haplotype	Frequency	GenBank Acc. No.	Origin	Reference
<i>C. langsdorfii</i>	<i>Clan1</i>	2	AB368693 JN412527	Kako River, Honshu, Japan Kako River, Honshu, Japan	Takada et al. (2010) present study
	<i>Clan2</i>	1	AB368690	Biwa Lake, Honshu, Japan	Takada et al. (2010)
	<i>Clan3</i>	1	AB368692	Urano River, Honshu, Japan	Takada et al. (2010)
	<i>Clan4</i>	1	AB368694	Shimanto, Shikoku, Japan	Takada et al. (2010)
	<i>Clan5</i>	1	AB368695	Tanegashima Island, Japan	Takada et al. (2010)
	<i>Clan6</i>	1	AB368683	Okinawa Island, Japan	Takada et al. (2010)
	<i>Clan7</i>	1	AB368686	Shigenobu, Shikoku, Japan	Takada et al. (2010)
	<i>Clan8</i>	1	AB368684	Urano River, Honshu, Japan	Takada et al. (2010)
	<i>Clan9</i>	4	DQ399920 DQ399921 DQ399922	Abashiri Lake, Hokkaido, Japan Abashiri Lake, Hokkaido, Japan Abashiri Lake, Hokkaido, Japan	Kalous et al. (2007) Kalous et al. (2007) Kalous et al. (2007)
			AB368688	Urano River, Honshu, Japan	Takada et al. (2010)
	<i>Clan10</i>	1	AB368687	Nagara, Honshu, Japan	Takada et al. (2010)
	<i>Clan11</i>	1	AB368689	Urano River, Honshu, Japan	Takada et al. (2010)
	<i>Clan12</i>	1	AB368685	Tanegashima Island, Japan	Takada et al. (2010)
	<i>Clan13</i>	4	DQ399930 DQ399932 EU186830 DQ868879	Chrudimka River, Czech Republic Chrudimka River, Czech Republic Lysimacheia Lake, Greece Amvrakia Lake, Greece	Kalous et al. (2007) Kalous et al. (2007) Tsipas et al. (2009) Tsipas et al. (2009)
	<i>Clan14</i>	1	AB368677	Taktsu, Honshu, Japan	Takada et al. (2010)
	<i>Clan15</i>	1	FJ169953	floodplain, Chomutov, Czech Republic	Papoušek et al. (2008)
	<i>Clan16</i>	3	JN412529 JN412530 AB368679	pool at Litvínovice, Czech Republic pool at Litvínovice, Czech Republic Okinawa Island, Japan	present study present study Takada et al. (2010)
	<i>Clan17</i>	3	DQ868878 DQ868877 DQ868876	Ozeros Lake, Greece Trichonida Lake, Greece Lysimacheia Lake, Greece	Tsipas et al. (2009) Tsipas et al. (2009) Tsipas et al. (2009)
	<i>Clan18</i>	1	AB368681	Amami-oshima Island, Japan	Takada et al. (2010)
	<i>Clan19</i>	1	AB368682	Tokunoshima Island, Japan	Takada et al. (2010)
	<i>Clan20</i>	1	AB368680	Okinawa Island, Japan	Takada et al. (2010)
<i>Clan21</i>	1	AB368678	Iki Island, Japan	Takada et al. (2010)	
<i>C. auratus</i>			EU663574 EU663599 EU663597	pet shop, Czech Republic Wuhan, Yangtze, China Nanking, Yangtze, China	Rylková et al. (2010) Rylková et al. (2010) Rylková et al. (2010)
	<i>C. gibelio</i>		EU663591 EU663594 HM000009	Cetina River, Bosnia and Herzegovina Canal de Fougères, Loire River, France Czerskie Rumunki, Poland	Rylková et al. (2010) Rylková et al. (2010) Kalous et al. (2012)
		<i>C. cuvieri</i>		JN402304	Lake Mikatako, Honshu
<i>C. carassius</i>			DQ399938 DQ399917	Milevsko, Elbe drainage, Czech Republic pond, Plon, Germany	Kalous et al. (2007) Kalous et al. (2007)
	<i>Cyprinus carpio</i>		HM008692	Mekong River, Thailand	Kalous et al. (2012)

during the 1000-year flood in 2002 (it is located at the inundation area). During this event ginbuna offspring could colonize many other locations in the Vltava catchment. Other fish could colonize the pools during the flooding but the apparently vanished and were not found in subsequent sampling during 2003 and 2006 (Kubečka, Okrouhlík, personal communication). These facts indicate that ecological impact of ginbuna on original ichthyofauna is probably significant and worth of further following up.

CONCLUSION

C. langsdorfii is most probably more widespread than has recently been known but its existence seems to remain hidden usually due to mistaken identity based on morphological similarity with the other species of the genus *Carassius*.

It seems to be important to gain more data for estimating its possible ecological impacts in newly inhabited areas, e.g. food competition, sexual parasitism or genetic contamination of native European populations of other *Carassius* species via hybridization (Hänfling et al., 2005). Unfortunately, until now the information on *C. langsdorfii* in European waters has been very limited.

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