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Phylogeny and biogeographic history of the cyprinid fish genus *Carassius* (Teleostei: Cyprinidae) with focus on natural and anthropogenic arrivals in Europe

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ABSTRACT

Freshwater fishes of the genus *Carassius*, widespread throughout Europe and Asia, are important aquaculture fishes and include the world's most important pet fish, the goldfish. The high morphologic similarity between the species, however, has up to now prevented reliable conclusions on their taxonomy, biogeography and introduction history. A phylogeny of the fish genus *Carassius* based on the cytochrome *b* sequence of 404 specimens collected from aquaculture and open water localities across Eurasia identifies most of the presently recognised species as monophyletic lineages, but also that at least one lineage exists that does not correspond to any described species. Within Europe, feral populations of *Carassius auratus* occur mainly in the Mediterranean area and Great Britain, while *Carassius gibelio* is found in most of non-Mediterranean Europe and some localities in Italy. *Carassius langsdorfii* has very scattered points of occurrence in at least six European countries. *C. auratus* and *C. langsdorfii* are not native to Europe. The populations of *C. gibelio* in eastern Central Europe and parts of Eastern Europe are considered as resulting from a natural postglacial range expansion, while the rest of Europe was colonised due to anthropogenic impact. The presence of diploid ($2n=100$) as well as triploid ($3n=150$) specimens in the three most widespread species indicates that ploidy level is not a character to identify the species of *Carassius*. A remarkably low genetic divergence in *C. gibelio* can be the result of clone selection in the gynogenetic populations. In general, our data present the first comprehensive overview about the genus *Carassius* in Europe based on genetic data.

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1. Introduction

The first and most important step in any study dealing with biological material is the proper identification of the investigated species, otherwise studies on biodiversity and distribution cannot be carried out, and these animals cannot be used as models in any type of investigation. The traditional and most practical way to identify fish species is based on morphological characters, but in cases of morphologically similar species genetic data can also help in the identification (Avise and Hamrick, 1996; Ogden, 2008).

Freshwater fishes of the genus *Carassius* are closely related to the common carp and include the goldfish, one of the best-known fishes at all. The genus occurs frequently across Eurasia from Portugal in the west to Japan in the east and from the Siberian Rivers in the north to southern China and Vietnam in the south (Szczerbowski, 2002a,b). They are farmed for aquaculture in East Asia (annual production in China about 2 million tonnes Gui and Zhou, 2010), Eastern Europe and Central Asia (FAO, 2011) and for this purpose they have been introduced from Asia to Europe several times (Savini et al., 2010). Especially the introduced forms from Asia are supposed to have a negative impact on European river ecosystems (Richardson et al., 1995). However, due to the morphological similarity of the species, the understanding of the taxonomy of the genus *Carassius* and the detailed distribution of species in Europe are poor. At present, five species are considered valid: *Carassius carassius* in most of Europe and western Asia, *Carassius langsdorfii* and *Carassius cuvieri* in Japan, *Carassius auratus* in China and *Carassius gibelio* in Europe (introduced lineages from Asia as well as native populations), Siberia and East Asia (Bănărescu, 1991; Hosoya, 2000; Szczerbowski, 2002a,b). Morphologically, *C. carassius* is the only species that can be identified easily (Kottelat and Freyhof, 2007), while the remaining species differ only slightly in morphological characters and will further on be referred to as the *C. auratus* complex.

The identification of species is even more complicated in many populations by the occurrence of specimens with different ploidy level (Abramenko et al., 1997; Jakovlić and Gui, 2011; Lusková et al., 2004). For a long time it has been believed that *C. gibelio* and *C. langsdorfii* are triploid ($3n=$ around 150), while the other species are diploid ($2n=100$); therefore the ploidy level has been taken as important character to identify species (Vasil'eva and Vasil'ev, 2000). In recent times it has

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been shown that diploid specimens also occur within *C. gibelio* and *C. langsdorfii* and triploid specimens have been found in *C. auratus* (Abramenko et al., 1997; Takada et al., 2010; Xiao et al., 2011). Triploid specimens are nearly all females and are considered to reproduce by gynogenesis after mating with any other cyprinid species (Cherfas, 1966; Flajšhans et al., 2008), but may incorporate sperm nuclei when coexisting with diploid bisexual populations of *Carassius* (Fan and Shen, 1990; Lamatsch and Stöck, 2009; Tóth et al., 2005; Zhou et al., 2000). Moreover, hybrids between *C. carassius* and *C. auratus* exist at least in Great Britain and can be difficult to identify (Hänfling et al., 2005).

The combination of morphologically similar species, of hybrid specimens and of gynogenetic lineages within species, hampers studies aiming to clarify the biogeography and taxonomy of the genus *Carassius*. Moreover, these fishes have a long history of introductions and translocations by man (Burmakin, 1963; Copp et al., 2005a). Their outstanding hardiness when transported in wet grass and a great possibility to survive and grow even in small ponds with eutrophic conditions made the species of *Carassius* one of the first candidates for stocking throughout and outside their natural distribution areas. In Europe and China, anthropogenic translocations of *Carassius* have occurred for hundreds of years (Balon, 2004). During Medieval times, monks developed an early carp aquaculture across Europe, which later developed into a flowering carp industry with global exchange of stocking material in the 19th and 20th century, and the unintended propagation and translocation of *Carassius* together with juvenile carps are until today a common side product of carp aquaculture worldwide (Copp et al., 2005a; Tóth et al., 2005). Moreover, the goldfish became the most popular pet fish of all times and was globally distributed, leading to uncounted events of release to open waters (Kottelat, 1997). Today, records of goldfish from open waters come from nearly all climatically suited parts of the world (Elvira, 2001; Kumar, 2000; Olden et al., 2008; Seegers et al., 2003). The fact that feral goldfish lose their orange colour and fancy fin shapes within a few generations and return to the wild phenotype has added to the problem of identification of species of the genus *Carassius*.

The distribution of species of *Carassius* in Europe and their translocations can be summarised as following: *C. carassius* occurs from the Rhine basin eastwards through most of Europe except of the Mediterranean basin. It has been introduced to the large part of Great Britain, Italy and France (Kottelat and Freyhof, 2007). *C. gibelio* was originally described from north-eastern Central Europe by Bloch (1782) but was at that time not mentioned or its status as species was doubted by ichthyologists in more western parts of Europe and the Danubian basin (Balon, 1962; Changeux and Pont, 1995; Holčík and Žitňan, 1978; Verreycken et al., 2007). Since 1940 it was recorded in the Danube River basin, first from Bulgaria and Romania, most likely as a result of introductions from the Russian part of the distribution area and since then has spread across most of Europe (Bănărescu, 1964; Drensky, 1948; Szczerbowski, 2002a,b). The goldfish *C. auratus* arrived as valuable pets in the 17th century to Portugal, France and Great Britain (Balon, 2004; Hervey and Hems, 1968; Kottelat, 1997). They were soon reproduced in captivity and spread across Europe. Distribution as ornamental species and stocking by private persons can be considered as a general and major way for the goldfish into open water systems (Copp et al., 2005b; Kottelat, 1997). Recently the Japanese species *C. langsdorfii* was discovered in the Elbe river system and Greece (Kalous et al., 2007; Takada et al., 2010; Tsipas et al., 2009).

In the present study, we identify more than 400 individuals of *Carassius* based on mitochondrial DNA and reconstruct their phylogeny. The ploidy level of a subsample of the analysed specimens of *Carassius* was estimated in order to test if the ploidy level is responsible for splits between species or lineages within species. Focusing on the relationships of the European populations we evaluate the number of introductions and the distribution of introduced species. On the base of the voluminous records of *Carassius* introductions and translocations, we reconstruct the biogeographic history of the introduced species of the *C. auratus* complex in Europe and report on the existence of at least one undescribed species in northeast Asia.

2. Material and methods

Altogether, 404 samples of *Carassius* from European, Asian and North American water bodies were included into the analysis. The dataset includes 183 new sequences, 121 sequences from our previous studies and 100 sequences obtained from GenBank. Our original samples were obtained via random sampling. As an outgroup, we used the common carp, *Cyprinus carpio*. Detailed information of sample origin and GenBank accession numbers are listed in Supplementary Table 1.

2.1. Reconstruction of phylogeny

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_F (GAA GAA CCA CCG TTG TTA TTC) and reverse primer Kai_R (ACC TCC RAY CTY CGG ATT ACA) (Šlechtová et al., 2006). Polymerase chain reaction (PCR) consisted of 3 µl template DNA, 3 µl of each primer, 15.5 µl of Combi PPP Master Mix (Top-bio) and ddH_2O up to 50 µl of reaction mix. The profile of the PCR, carried out on an MJ Mini™ thermocycler (Bio-Rad), started with initial denaturation at 95 °C for 2 min followed by 35 cycles consisting of denaturation step at 95 °C for 1 min, annealing step at 52 °C for 30 s and elongation at 72 °C for 30 s; the last step was final elongation at 72 °C for 10 min.

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

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 and Ronquist, 2001).

2.2. Ploidy level determination

Measurements of ploidy level followed the method described by Lamatsch et al. (2000) for fixed fin clips. Heparinised red blood cells from female chicken (*Gallus gallus*) cells were used as the internal standard (genome size 2.5 pg/nucleus; Vinogradov, 1998).

Ethanol fixed fin clips were minced in 2.1% citric acid/0.5% Tween 20 and incubated for 15 min with gentle shaking at room temperature (RT). Fish cells and 100 µl of the chicken erythrocyte solution in phosphate buffered saline (PBS) were centrifuged for 5 min at 300 ×*g*, the supernatant was discarded and the cell pellets were resuspended in 400 µl 0.5% pepsin in 0.1 M HCl. After incubating at RT for 10 min with gentle shaking, the cells were stained overnight at 4 °C by adding 1100 µl DAPI solution (5.9% citric acid trisodium salt $2H_2O$, 0.0002% DAPI). Immediately before analysis the fish samples were filtered through a 50 mm nylon mesh (Celltrics, Partec©), to prevent obstruction of the flow chamber by fin rays. To certify that nuclei were stained completely, 1:10 dilutions with DAPI were used for measurements. For each measurement fish fin clip cells and chicken red blood cells were mixed in such a way that similar final concentrations were obtained. All measurements were conducted on a Partec Ploidy Analyser PA-II applying a mercury lamp (Partec, Münster, Germany). At least 10,000 cells were measured per sample.

Ploidy level was determined for 128 specimens (67 samples original to this study). Cluster analysis based on K-means clustering method

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