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MORPHOLOGICAL AND mtDNA SEQUENCE STUDIES SEARCHING FOR THE ROOTS OF SILVER CRUCIAN CARP *CARASSIUS GIBELIO* (CYPRINIDAE) FROM PONDS OF SERGIEVKA PARK, SAINT PETERSBURG, RUSSIA

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ABSTRACT

The roots of the silver crucian carp *Carassius gibelio* from ponds of Sergievka Park (Petergof, Saint Petersburg), were surveyed based on morphological and molecular (mitochondrial DNA sequences, control and cytochrome *b* regions) study of samples of this species, collected there in 1930-thies and 2008, in comparison with *C. g. gibelio* and *C. gibelio*. subsp. M. collected from Kazakhstan. It is demonstrated, that the fish is most similar to *C. gibelio gibelio* morphologically. Their mitochondrial haplotypes were clustered with those of the Far Eastern populations such as the Amurian one. The result indicates that the silver crucian carp of Sergievka Park has been introduced originally from the Far East region.

Key words: crucian carp, morphology, mtDNA, Prussian carp

ИЗУЧЕНИЕ МОРФОЛОГИИ И ПОСЛЕДОВАТЕЛЬНОСТИ мтДНК СЕРЕБРЯНОГО КАРАСЯ *CARASSIUS GIBELIO* (CYPRINIDAE) ИЗ ПРУДОВ ПАРКА СЕРГИЕВКА (САНКТ-ПЕТЕРБУРГ, РОССИЯ) С ЦЕЛЬЮ ВЫЯСНЕНИЯ ЕГО ПРОИСХОЖДЕНИЯ

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РЕЗЮМЕ

Изучено происхождение *Carassius gibelio*, интродуцированного в пруды парка Сергиевка (Петергоф, Санкт-Петербург) на основе сравнительно-морфологического и молекулярно-генетического (участки контрольного региона и цитохрома *b* мтДНК) изучения выборок серебряного карася, собранных в этих прудах в 1930-х и 2008 гг., в сравнении с *C. gibelio gibelio* и *C. gibelio* subsp. M из водоемов вблизи г. Семипалатинска (Казахстан). Показано, что по морфологическим признакам рыбы из прудов парка Сергиевка наиболее сходны с *C. gibelio gibelio*. Полученные гаплотипы *C. gibelio* сходны с таковыми карасей из реки Амур и других рек Дальнего Востока. Следовательно, серебряный карась вселен в пруды парка Сергиевка из водоемов Дальневосточного региона России.

Ключевые слова: серебряный карась, морфология, мтДНК, прусский карась

INTRODUCTION

Crucian carp, the genus *Carassius* Nilsson, 1832 is well known for its controversial taxonomy and contentious morphological classification (Berg 1949; Nakamura 1969). At least three major species have been recognized: common crucian carp *C. carassius* (Linnaeus, 1758), mainly from Europe; silver crucian carp *C. auratus* (Linnaeus, 1758), mainly from Asia; and Japanese crucian carp *C. cuvieri* Temminck et Schlegel, 1846 from Lake Biwa, Japan (Berg 1949; Nakamura 1969; Hosoya 2002). The main taxonomic controversy has centered on *C. auratus*, because of its variation in morphology as well as in ploidy size (Nakamura 1969). In the Eurasian Continent, however, two sub-species have been recognized traditionally: Prussian carp *C. auratus gibelio* (Bloch, 1782) from Germany and Poland to the Amur River system, and *C. auratus auratus* from China (Berg 1949; Wu 1977), with some authors treating them as the same subspecies, *C. auratus gibelio* and some as distinct species (Hensel 1971; Kottelat and Freyhof 2007; Kalous et al. 2007).

Because the species name "*auratus*" (Linnaeus 1758) was established based on the domestic goldfish with a tri-lobed tail, International Commission on Zoological Nomenclature (2003) has recommended that the name should be limitedly applied to the goldfish. Sakai et al. (2009) have discussed that the name "*gibelio*" proposed by Bloch (1782) from Prussia should be used for the wild form instead of "*auratus*". However, it has been claimed that ongoing introductions of Far Eastern crucian carp to Europe have brought about considerable propagation of the Far Eastern form in European waters (Hensel 1971; Holčík and Žitňan 1977; Cherfas 1979), resulting in much difficulty in proving the existence of native European Prussian carp (Hensel 1971). Moreover, the type specimen of *Cyprinus gibelio* Bloch (1782) has been lost (Paepke 1999) and it is now difficult to judge which type of morphology should be identified as the original "*gibelio*", the question having been unsolved yet.

By the way, Sakai et al. (2009) have found a new stock of silver crucian carp from Kazakhstan, tentatively called *C. gibelio* subsp. M, with a peculiar mitochondrial haplotype different from those ever known and with an intermediate number of gill-rakers between *C. auratus* or *gibelio* and *C. carassius*. They suggested its wider distribution in Central Eurasia such as from the Lena River system (Kirillov 1956) to Saint Petersburg (Grib 1935) based on the morphological characteristics described. They also discussed a possibility that *C. gibelio* subsp. M may represent the true Prussian carp *C. gibelio* described by Bloch (1782), if the fish from European Russia appears to be the same stock with *C. gibelio* subsp. M.

Recently, Takada et al. (2010) conducted a comprehensive mitochondrial gene sequence analysis of *C. auratus* or *gibelio*. They revealed the existence of two mitochondrial superlineages; one including three lineages from Japan (clades I–III) and the other including four, one from the Ryukyu Islands (clade IV), another from Taiwan Island (clade VI) and the other two from Eurasian Continent (clades V and VII). Among them, one of the Eurasian lineages (clade V) contained a monophyletic cluster of three cytochrome b sequences recorded from the Czech Republic. Takada et al. (2010) considered them to be native to Europe, although all the other fish registered from outside East Asia were judged as introduced from there. However, they did not touch *C. gibelio* subsp. M sensu Sakai et al. (2009), and then it is still unknown whether or not the same stock as *C. gibelio* subsp. M is distributed in other regions than Kazakhstan, especially in Europe.

The authors collected crucian carp from ponds of Sergievka Park (Petergof, Saint Petersburg), where Grib (1935) reported the fish that is morphologically similar to *C. gibelio* subsp. M sensu Sakai et al. (2009). Subsequent studies aim to determine the roots of the fish from Sergievka Park based on morphological characters and mitochondrial DNA (mtDNA) sequence analyses, anticipating a discovery of the true Prussian carp. The consequence of the research is that the fish seem not to be native, but to have been introduced stock possibly from the Far East region.

MATERIAL AND METHODS

Fish collection. Silver crucian carp were collected from ponds of Sergievka Park initially settled in 18th century (Vlasov 2005) in 2008 (Fig. 1). The fish were initially identified following the keys given by Berg (1949), being 24 individuals of *C. gibelio* (Fig. 2A). Three individuals of common crucian carp *C. carassius* were also collected but released soon after. Immediately after collection, nine fish were anesthetized and a drop of blood from the first gill arch was placed on a slide for measuring erythrocyte size. A piece of pectoral fin was clipped and fixed

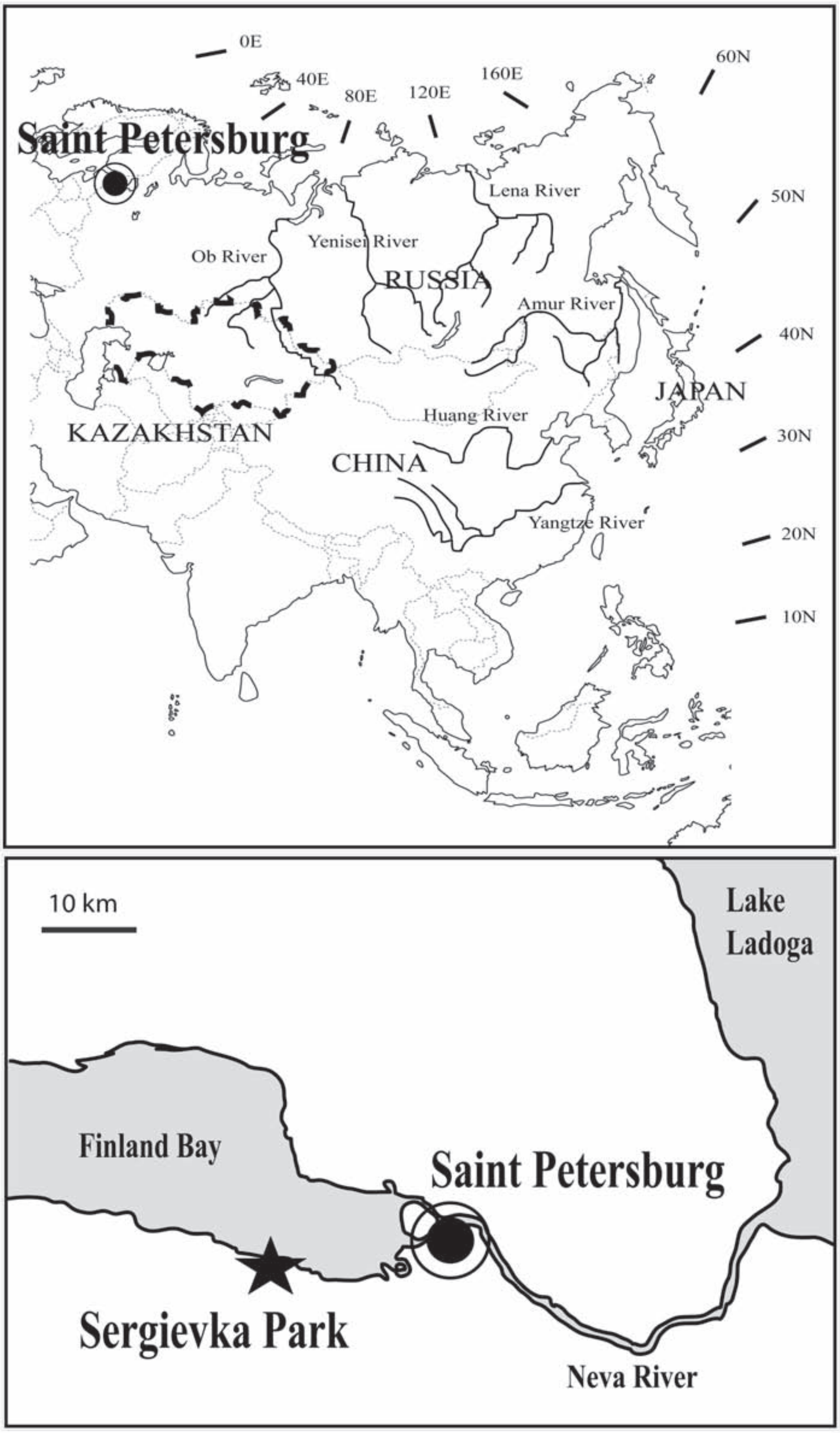

Fig. 1. Map showing the location of Sergievka Park, Saint Petersburg, Russia.

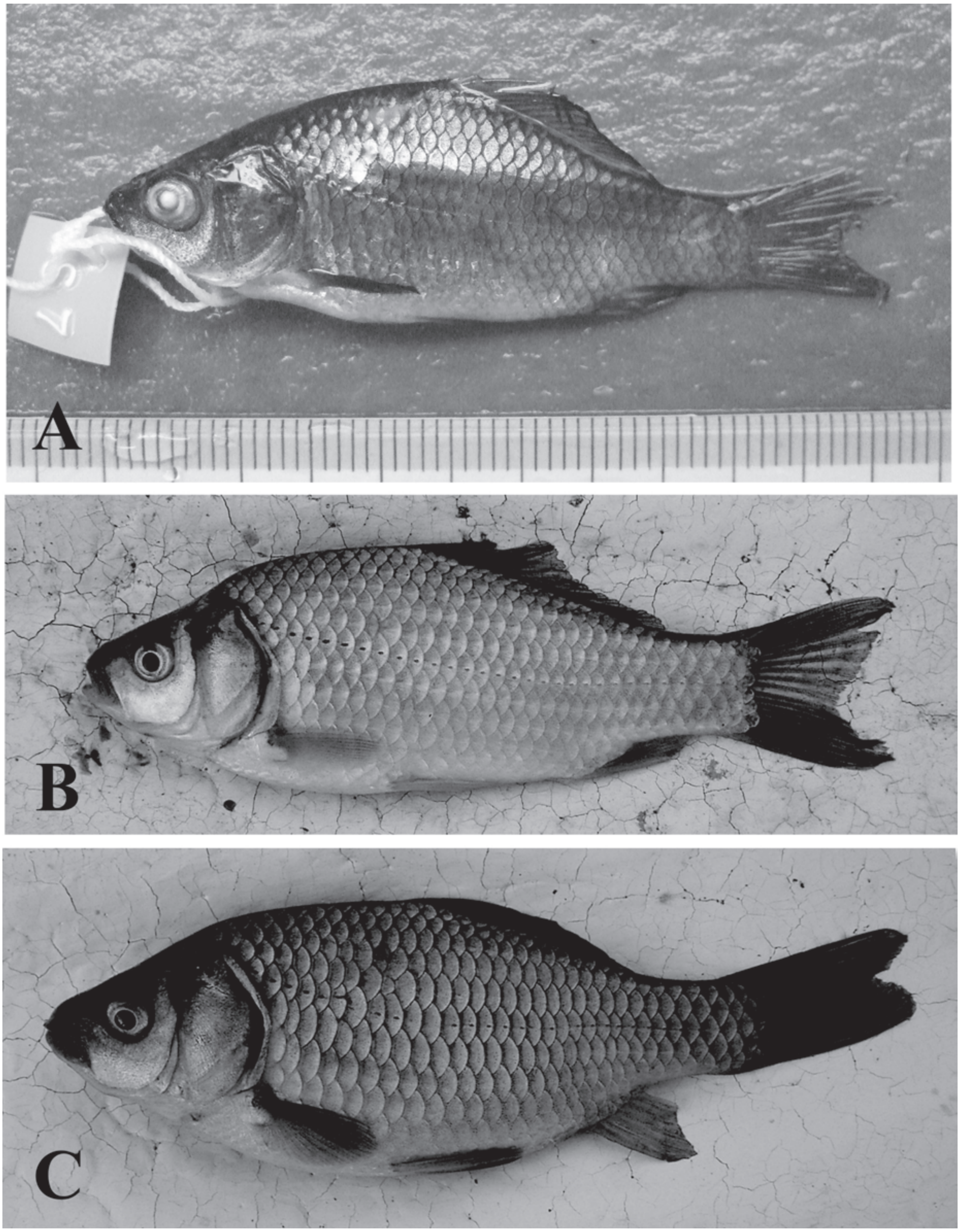

Fig. 2. Specimens of *Carassius gibelio*: (A) *C. gibelio* collected from Sergievka Park, 44.9 mm standard length (SL); (B) *C. g. gibelio* from Kazakhstan, 95.5 mm SL; (C) *C. g.* subsp. M from Kazakhstan, 98.8 mm SL.

Fig. 3. Three specimens of *Carassius gibelio*, 110.8–125.6 mm SL, collected from Sergievka Park by Grib (1935).

Table 1. Samples of silver crucian carps genus *Carassius* from Sergievka Park (Russia), and Kazakhstan. Designations: * – significant difference from fish collected from Sergievka Park in the present study ($p < 0.05$, Student's t-test); ZIN – catalogue number registered in Zoological Institute of the Russian Academy of Sciences; HUMZ – catalogue number registered in Hokkaido University, Laboratory of Marine Zoology, Faculty of Fisheries. For other designations see Material and Methods.

No. ind.	Sergievka Park		Kazakhstan (Sakai et al. 2009)	
	Present study	Grib (1935)	*C. gibelio gibelio*	*C. g.* subsp. M
	24	3	19	20
SL (mm)	31.3–52.4	110.8–125.6	66.1–127.9	42.9–104.5
	In % of SL (mean with standard deviation in parenthesis)			
BD	41.1 (3.2)	47.8 (1.7)	30.2 (2.3)	40.3 (1.7)
HL	33.2 (1.3)	31.3 (2.5)	30.4 (1.4)	32.1 (1.1)
CPD	15.2 (0.7)	16.0 (0.3)	15.1 (0.8)	14.1 (0.8)
PreDL	54.0 (2.2)	–	52.8 (1.8)	54.6 (2.2)
PrePL	52.7 (1.9)	–	50.9 (1.6)	53.2 (1.5)
PreAL	78.2 (2.0)	–	77.3 (2.6)	78.0 (1.9)
	In % of HL			
SnL	29.4 (2.2)	30.6 (2.5)	24.6 (1.6)	24.7 (1.5)
ED	28.4 (6.3)	17.8 (1.3)	21.0 (2.0)	21.4 (2.0)
UJL	23.8 (2.2)	–	21.6 (1.6)	24.9 (1.7)
V	29.4 (0.6)	–	30.4 (0.5)*	30.9 (0.6)*
D	16.4 (0.7)	15.0 (0.0)*	17.0 (0.5)*	15.6 (0.7)*
A	5.0 (0.0)	5.0 (0.0)	5.0 (0.0)	5.0 (0.0)
Ll	28.0 (0.8)	32.5 (0.7)*	30.3 (0.5)*	30.6 (0.8)*
Sal	6.1 (0.6)	6.0 (0.0)	5.9 (0.4)	5.0 (0.0)*
Sbl	5.2 (0.4)	5.0 (5.0)	5.3 (0.5)	4.9 (0.3)*
Gr	36.9 (1.5)	45.0 (2.8)*	46.7 (1.1)*	38.5 (2.1)*
ZIN	–	25220	54385	54384
HUMZ	209617–209640	–	209587–209601	209572–209586

in 99% ethanol for DNA extraction. The remaining bodies and the rest of the fish were labeled and fixed in 70% ethanol for morphological observation.

Morphological analyses. Meristics, known to be variable among both species and sub-species (Nakamura 1969), were selected as follows: vertebrae, inclusive of Weberian apparatus (*V*), branched dorsal fin rays (*D*), branched anal fin rays (*A*), scales on lateral line series (*Ll*), scales on transverse series from dorsal fin base to above lateral line (*Sal*), scales on transverse series from anal fin base to below lateral line (*Sbl*), and gill rakers on first gill arch (*Gr*). Morphometrics were measured as follows: standard length (*SL*), body depth (*BD*), head length (*HL*), caudal peduncle depth (*CPD*), pre-dorsal length (*PreDL*), pre-anal length (*PreAL*), snout length (*SnL*), eye diameter (*ED*), and upper jaw length (*UJL*).

The fish from the same locality collected by Grib (1935; Fig. 3) and *C. gibelio gibelio* (Fig. 2B) and *C. gibelio* subsp. M (Fig. 2C) from Kazakhstan collected by Sakai et al. (2009) were morphologically compared with the present fish, their catalogue numbers being indicated in Table 1. Difference from the fish of Sergievka Park in meristics, which are possibly hardly affected by relative growth, was tested by Student's t-test.

Erythrocyte diameter of ten cells (long and short axes) of each fish was measured utilizing a micrometer attached to a microscope, and mean μm^2 was calculated, such possibly being an indicator of relative

Table 2. Samples of crucian and common carps used for mtDNA analysis. Designation: * – accession number in DDBJ/GenBank/EMBL DNA Data Bank.

Access. N.*	Locality	Literature
	Control region	
	***Carassius gibelio* from Saint Petersburg**	
AB587672	Sergievka Park, Saint Petersburg, Russia	Present study
	***C. gibelio* subsp.**	
AB274413	Mikhailovski Lake, Kazakhstan	Sakai et al. (2009)
	C. gibelio gibelio	
AB274414	Chara River, Kazakhstan	Sakai et al. (2009)
AB274415	Chara River, Kazakhstan	Sakai et al. (2009)
AB274416	Chara River, Kazakhstan	Sakai et al. (2009)
AB080012	Amur River, Russia	Yamamoto et al. (2010)
AB080013	Amur River, Russia	Yamamoto et al. (2010)
AB080014	Amur River, Russia	Yamamoto et al. (2010)
AY940117	Labe River, Czech Republic	Mendel et al. (unpubl.)
AY940118	Marava River, Czech Republic	Vetesnik et al. (2007)
AY940119	Marava River, Czech Republic	Mendel et al. (unpubl.)
AJ388413	France	Gilles and Lecointre (unpubl.)
	C. gibelio langsdorfii	
AB079925	Kasumigaura Lake, Japan	Iguchi et al. (2003)
AB079905	Biwa Lake, Japan	Yamamoto et al. (2010)
	C. gibelio grandoculis	
AB079901	Biwa Lake, Japan	Yamamoto et al. (2010)
AB079903	Biwa Lake, Japan	Yamamoto et al. (2010)
	C. gibelio buergeri	
AB079971	Kako River, Japan	Yamamoto et al. (2010)
AB079965	Chikugo River, Japan	Yamamoto et al. (2010)
	***C. gibelio* subsp. 1**	
AB079951	Urano River, Japan	Yamamoto et al. (2010)
AB079940	Mikata Lake, Japan	Yamamoto et al. (2010)
	***C. gibelio* subsp. 2**	
AB079921	Sakasazawa River, Japan	Iguchi et al. (2003)
AB079929	Kasumigaura Lake, Japan	Iguchi et al. (2003)
	***C. gibelio* subsp. A (traditionally named *C. auratus auratus*)**	
AB080009	Yangtze River, China	Yamamoto et al. (2010)
AB080010	Yangtze River, China	Yamamoto et al. (2010)
AB080011	Yangtze River, China	Yamamoto et al. (2010)
	C. cuvieri	
AB080015	Biwa Lake, Japan	Iguchi et al. (2003)
	***C. auratus* (Goldfish)**	
AB052332	Fish dealer	Murakami et al. (2001)

Table 2. Continued.

Access. N.*	Locality	Literature
	C. carassius	
AB274417	Mikhailovski Lake, Kazakhstan	Sakai et al. (2009)
DQ007649	Marava River, Czech Republic	Mendel et al. (unpubl.)
	***Cyprinus carpio* (Common Carp)**	
X61010	Taiwan	Chang et al. (1994)
	Cytochrome *b*	
	***Carassius gibelio* from Saint Petersburg**	
AB605597	Sergievka Park, Saint Petersburg, Russia	Present study
	C. gibelio gibelio	
AB368700	Amur River, Russia	Takada et al. (2010)
AB368701	Amur River, Russia	Takada et al. (2010)
AB368707	Ryukyu Islands	Takada et al. (2010)
GU170401	Amur River, China	Liang et al. (unpubl.)
DQ399926	Elbe River, Czech Republic	Kalous et al. (2007)
DQ399935	Elbe River, Czech Republic	Kalous et al. (2007)
DQ399940	Elbe River, Czech Republic	Kalous et al. (2007)

ploidy size. The data of *C. carassius* (diploidy) and *C. gibelio* subsp. M (triploidy) from Kazakhstan (Sakai et al. 2009) were used for comparative purpose.

Sex was determined by a visual examination of the gonads.

Mitochondrial analyses. Total genomic DNA was extracted from the fin samples, which were digested by proteinase K and purified by standard phenol/chloroform extraction. The first one-third of the control region and the cytochrome *b* region of mtDNA were amplified with primers devised by Iguchi et al. (2003) and Takada et al. (2010), respectively. PCR amplification was conducted according to standard protocols with GeneAmp PCR 9700 (Applied Biosystems Inc., Foster City, CA, USA). Amplified double-stranded DNA was sequenced on an automated DNA sequencer ABI 310 (Applied Biosystems Inc., Foster City, CA, USA) with the amplification primers using the BigDye Terminator cycle sequencing kit ver. 3.1 (Applied Biosystems Inc., Foster City, CA, USA). Registered accession numbers in the DDBL/GenBank/EMBL DNA Data Bank were shown in Table 2.

To determine the relationships between the present crucian carp and those from other regions, a phylogenetic analysis was conducted incorporating data already registered with the DDBJ/GenBank/EMBL DNA Data Bank, most of which are the same data already been analyzed by Sakai et al. (2009) and Takada et al. (2010) (Table 2). Alignment of multiple sequences was done using Clustal W 1.6 (Thompson et al. 1994). A neighbor-joining tree (Saitou and Nei 1987) with 1000 bootstrap replications (Efron 1979) was constructed using PAUP* 4.0b10 (Swofford 2002), based on 283 bp excluding insertions/deletions for the control region and 1141 bp for the cytochrome *b* region, with the model of Hasegawa et al. (1985) selected by ModelTest ver. 3.7 (Posada and Crandall 1998).

RESULTS

Morphological differences. Morphological comparison is shown in Table 1. The fish from Sergievka Park were the smallest of the samples, and relative measurements such as BD, HL, ED, etc. were differ-

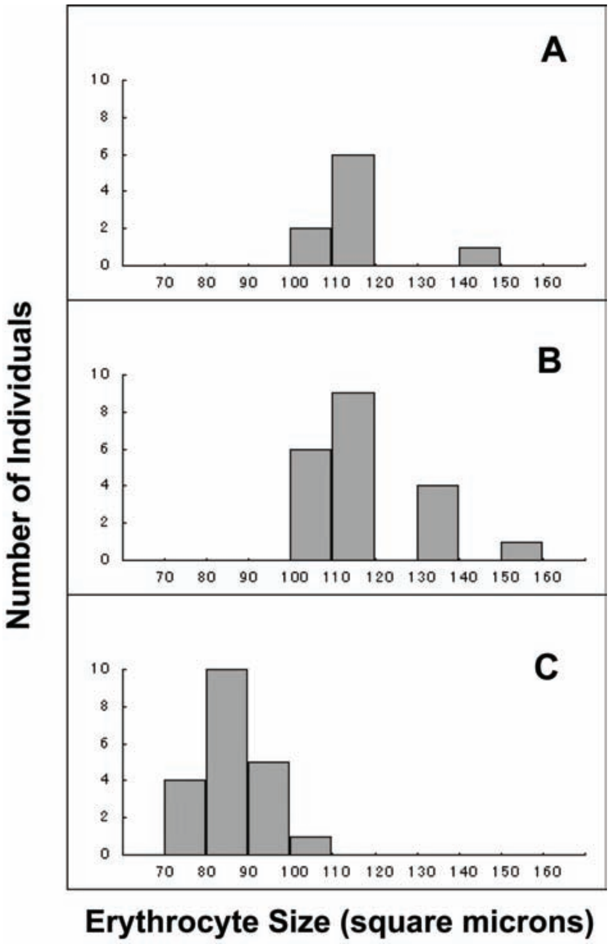

Fig. 4. Erythrocyte size (square microns) of *Carassius gibelio* from Sergievka Park (A), *C. g. gibelio* (B) and *C. carassius* (C) from Kazakhstan.

ent even from those of the same locality collected by Grib (1935).

Meristics of the fish from Sergievka Park, except for anal fin rays (*A*), were significantly different from *C. gibelio* subsp. M from Kazakhstan. Dorsal fin rays (*D*), gill rakers (*Gr*) and lateral line scales (*Ll*) were also different from those of all the other samples. On the other hand, transverse scales above (*Sal*) and below the lateral line (*Sbl*) were not different from the fish by Grib (1935) as well as *C. gibelio gibelio* from Kazakhstan.

Erythrocyte size and sex. The mean erythrocyte sizes were more than 100 μm^2 in the fish from Sergievka Park and *C. gibelio* subsp. M from Kazakhstan, and less than 110 μm^2 in *C. carassius* from Kazakhstan (Fig. 4A, 4B, 4C, respectively).

All nine individuals of fish from Sergievka Park were too young to determine their sex by eyes, whereas all 20 individuals of *C. gibelio* subsp. M from Kazakhstan were females and six out of 20 *C. carassius* from Kazakhstan were males.

Phylogenetic analysis. The haplotypes of *C. carassius* formed a species cluster based on control region sequences (Fig. 5). The remaining haplotypes were included in the *C. gibelio* group (bootstrap probability 76%), being divided into three; *C. gibelio* subsp. M from Kazakhstan, CHINA-AMUR (87%) and JAPAN clusters (less than 50%). The CHINA-AMUR cluster was subdivided into two clusters, one mainly from China (96%, clade VII by Takada et al. 2010) and the other mainly from Amur (99%, clade V by Takada et al. 2010), the fish from Sergievka Park being included in the latter.

According to the result based on cytochrome *b*, the sequence of fish from Sergievka Park was just the same as that of one haplotype from Czech Republic (Fig. 6). Moreover, the sequence registered from the Chinese Amur was also the same as them. The three sequences recorded from Czech Republic, consequently, did not form their own cluster anymore.

DISCUSSION

The silver crucian carp from ponds of Sergievka Park was morphologically more similar to *Carassius gibelio gibelio* than *C. gibelio* subsp. M sensu Sakai et al. (2009) from Kazakhstan, whereas the number of gill rakers was smaller than the former and near to the latter. The number of gill rakers in cyprinids is known to increase according to growth (Nakamura 1969). The smaller number of gill rakers in the present sample, therefore, is possibly due to their smaller body size.

Erythrocyte size shows that the fish from Sergievka Park are possibly triploid (or more) as *C. gibelio* subsp. M from Kazakhstan in comparison with that of diploid *C. carassius*. Such triploid populations are rather common wherever silver crucian carp exists (Cherfas 1979).

Phylogenetic analysis indicates that the fish from Sergievka Park is not native but is possibly introduced stock originated from the Far East region. In fact, the introductions of the Far Eastern crucian carp to Europe via Russian waters have brought about considerable propagation of them in there (Hensel 1971; Holčík and Žitňan 1977; Cherfas 1979). The

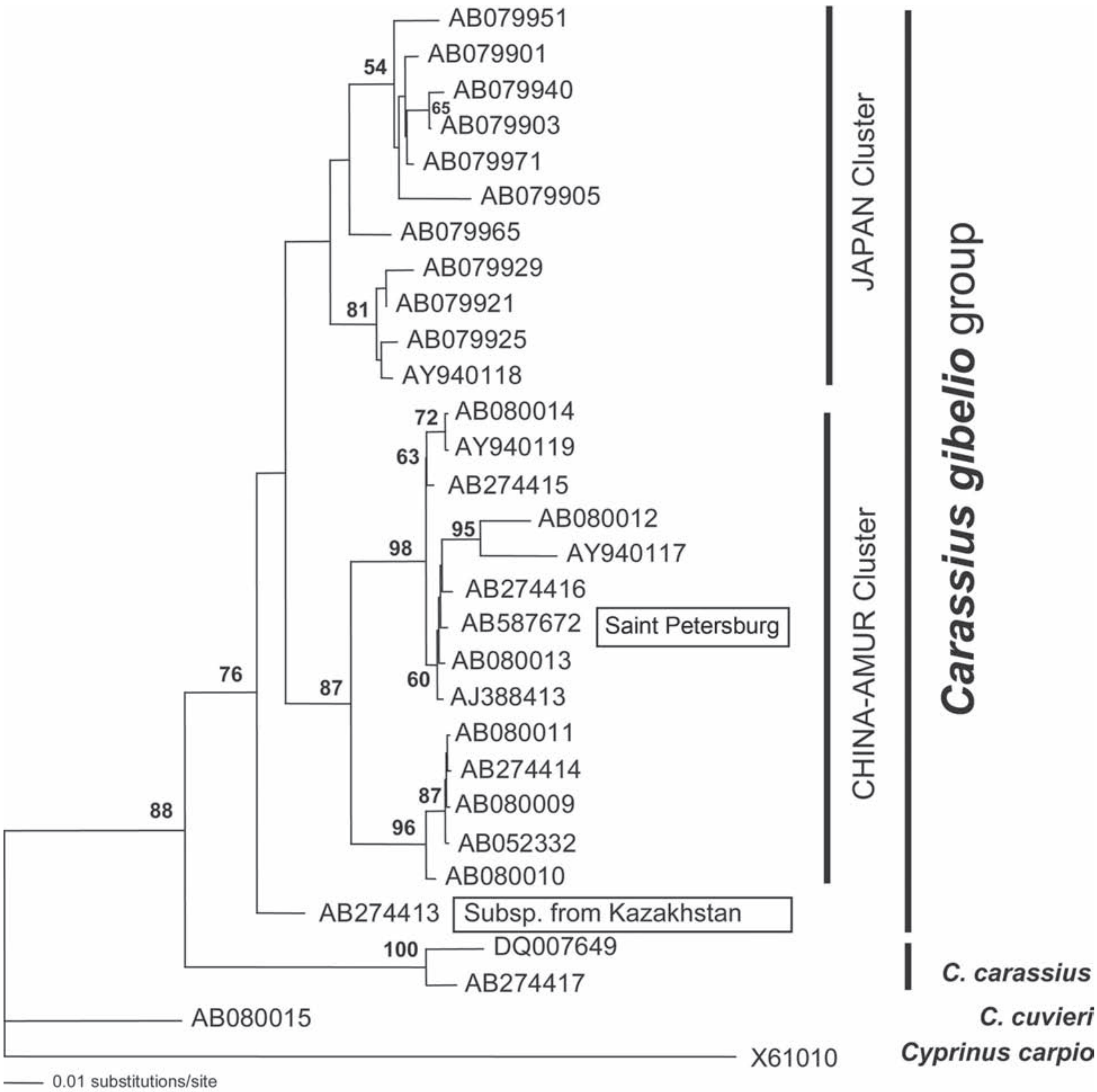

Fig. 5. Mitochondrial DNA phylogeny of crucian carps based on control region sequences by the neighbour-joining method with % bootstrap probabilities (1000 replications). See Table 2 for OUT names. Enclosed haplotypes are those of *Carassius gibelio* from Sergievka Park, Saint Petersburg, and *C. g.* subsp. M from Kazakhstan.

fish of Sergievka Park must have been one of such. According to Takada et al. (2010), two lineages exist in the Eurasian Continent, clade V and VII. One of them, clade V contained a monophyletic cluster of cytochrome *b* sequences from the Czech Republic, being considered to be native to Europe (Takada et al. 2010). The fish from Sergievka Park of the present study, however, showed just the same cytochrome *b* sequence as that of one haplotype of the Czech Republic cluster as well as that registered from the Amur River system. The cluster recorded from Czech Republic, consequently, is not unique to Europe

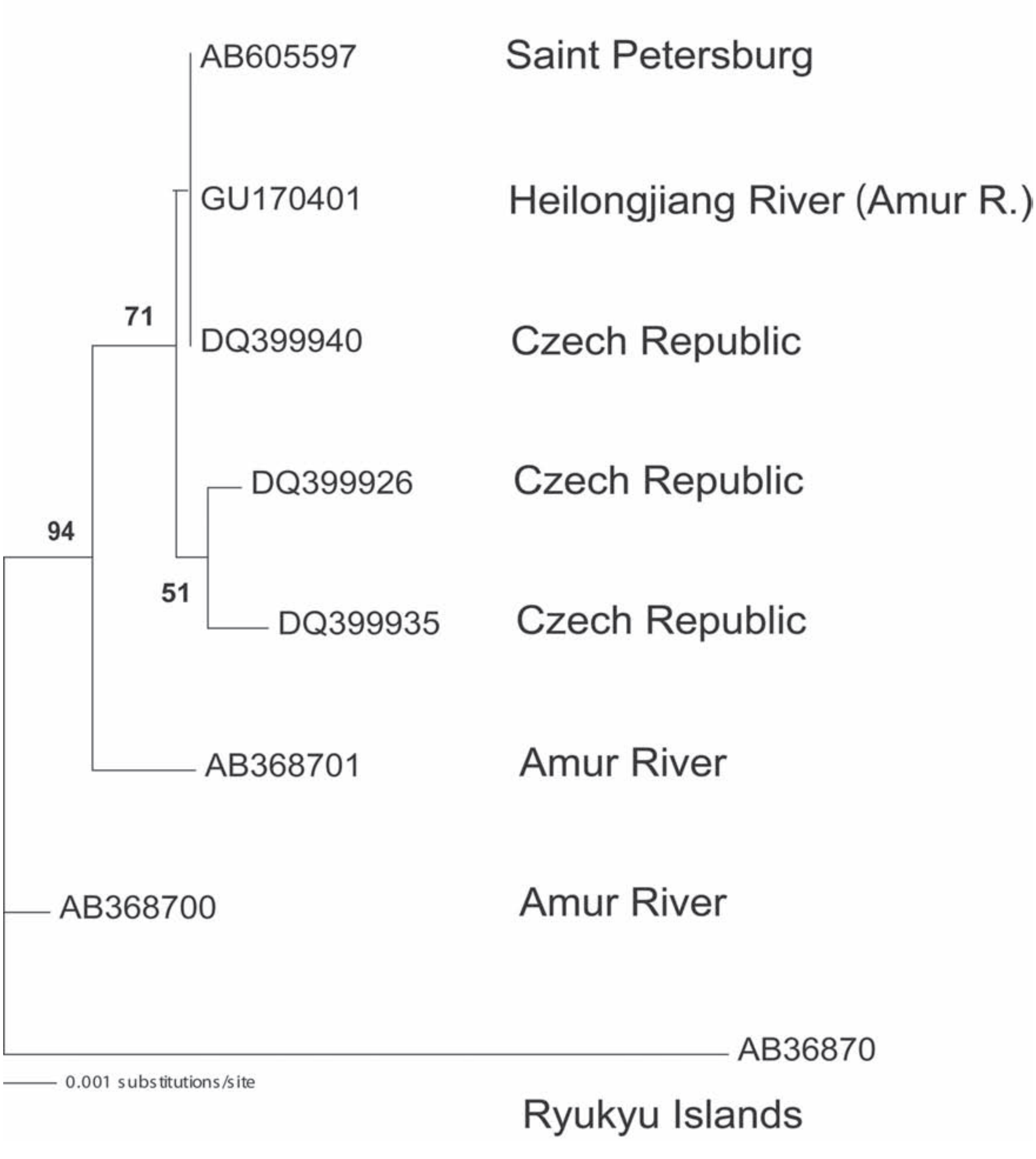

Fig. 6. Mitochondrial DNA phylogeny based on cytochrome *b* sequences by the neighbour-joining method with % bootstrap probabilities (1000 replications). See Table 2 for OUT names. Enclosed haplotype is that of *Carassius gibelio* from Sergievka Park, Saint-Petersburg. The haplotype from Ryukyu Islands (AB36870) is a member of clade VII, possibly being non-native to Ryukyu's, and the other haplotypes are members of clade V sensu Takada et al. (2010).

anymore. It would support the Amurian origin of the Czech fish as well as the Sergievka fish.

According to Bychowsky (1933), crucian carp had possibly been introduced to the ponds of Sergievka Park around 1913, but he did not describe from where. Grib (1935) himself also suggested the possibility that the fish had been introduced from Asia. Chmilevsky and Ivanov (2005) listed eight fish species inhabiting the ponds of Sergievka Park, including *Percottus glehni* that is also a member of the Amurian fish fauna (Berg 1949). Such introductions of alien species to the ponds may have been done several times.

Sakai et al. (2009) reported a new crucian carp stock *C. gibelio* subsp. M, seemingly native to Kazakhstan. Under the ambiguity of what the Bloch's (1782) "*gibelio*" is, they also discussed a possibility that the Kazakhstan fish may represent the true Prussian carp *C. gibelio* by Bloch (1782), if the fish from European Russia such as from Sergievka Park documented by Grib (1935) appears to be the same stock with *C. gibelio* subsp. M. However, morphological and genetic data clearly reject that possibility. The silver crucian carp from Sergievka Park is thought to have been an introduced stock originated from the Far East region such as the Amur River. It is still unknown, therefore, whether *C. gibelio* native to Europe exists or not.

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