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Significant genetic differentiation between native and introduced silver carp (*Hypophthalmichthys molitrix*) inferred from mtDNA analysis

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Abstract Silver carp *Hypophthalmichthys molitrix* (Cyprinidae) is native to China and has been introduced to over 80 countries. The extent of genetic diversity in introduced silver carp and the genetic divergence between introduced and native populations remain largely unknown. In this study, 241 silver carp sampled from three major native rivers and two non-native rivers (Mississippi River and Danube River) were analyzed using nucleotide sequences of mitochondrial COI gene and D-loop region. A total of 73 haplotypes were observed, with no haplotype found common to all the five populations and eight haplotypes shared by two to four populations. As compared with introduced populations, all native populations possess both higher haplotype diversity and higher nucleotide diversity, presumably a result of the founder effect. Significant genetic differentiation was revealed between native and introduced populations as well as among five sampled populations, suggesting strong selection pressures might have occurred in introduced populations. Collectively, this study not only provides baseline information for sustainable use of silver carp in their native country (i.e., China), but also offers first-hand genetic data for the control of silver carp in countries (e.g., the United States) where they are considered invasive.

Keywords Silver carp · Native and introduced populations · Genetic diversity · Divergence · Mitochondrial DNA

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Introduction

The silver carp (*Hypophthalmichthys molitrix*) is native to China, with a natural range extending from the Pearl River in the south to the Amur River in the north. Silver carp are thought to originate from the Yangtze-Yellow eastern plain of China in the Pliocene (about 350 million years ago) (Li and Fang 1990). Approximately 110,000 years ago during the Pleistocene, silver carp arrived at the Amur River through the Liaohe River, and arrived at the Pearl River through the Yangtze River and the Qiantang River. Within its native

region, different populations of this species have developed through geographic isolation, adaptation, and accumulation of mutations.

Silver carp have been one of the most important aquaculture species in China for over a thousand years (Li and Mathias 1994). Since the 1950s, it has been introduced into at least 88 countries and territories for various reasons including aquaculture, capture fisheries enhancement, and plankton control (Kolar et al. 2007). In China and in many parts of its introduced ranges, silver carp are an integral part of fish culture and are an important source of protein for human consumption (Li and Mathias 1994). In North America, they are considered a highly undesirable invasive species (Conover et al. 2007; Kolar et al. 2007) and many dedicated efforts have been made to prevent the invasion and establishment of silver carp in the Laurentian Great Lakes (Asian Carp Workgroup 2010).

As an important economic species, the genetic diversity and variation of the silver carp have been extensively studied using various genetic markers, including isoenzyme (Zhao and Li 1996; Jiang et al. 1998), mitochondrial DNA RFLP (Lu et al. 1997; Zhang et al. 2002), RAPD (Zhang et al. 1999, 2002) and SSR (Lu and Sun 2005; Liao and Yang 2006; Zhu et al. 2007). However, most studies have focused on silver carp populations from the Yangtze River or have been limited within the native region of its distribution (Zhao and Li 1996; Lu et al. 1997; Zhang et al. 1999; Liao et al. 2002; Zhu et al. 2007). Furthermore, the extent of genetic diversity in introduced populations and genetic divergence between native and introduced populations remains unknown. It is thus important to perform systematic genetic analysis among populations from the major native and introduced river systems.

Mitochondrial DNA (mtDNA), because of its characteristics of maternal inheritance, relatively fast evolution rate of mutation, and lack of recombination, has been used as a useful molecular marker for population genetics. The mitochondrial cytochrome oxidase subunit I (COI) gene has been widely used in fish phylogenetic analysis because of its moderate mutation rate and ability to differentiate at the population and species levels (Yang and Zhang 2007; Niu and Li 2008; Guo and Yu 2009; Peng and Wang 2009). As the fastest evolving mtDNA marker, the control region (D-loop) has been commonly used in population genetic and biogeographical studies (Wang et al. 2006; Li and Chen 2008; Zhu et al. 2008; Peng and Dai 2009). In this study, we sequenced the mitochondrial D-loop region and the mitochondrial COI gene of silver carp samples collected from five major rivers or river basins over the world to study population genetic structure of silver carp and estimate genetic change between native and introduced populations. This result will provide baseline information for sustainable use of silver carp resources in their native environments or control of silver carp where they are considered invasive.

Materials and methods

Sample collection

A total of 241 silver carp specimens were collected from rivers in their native country (Yangtze, Pearl and Amur Rivers in China) and from rivers in countries where silver carp have been introduced (the Mississippi River basin in the United States and the Danube River in Hungary) from 2005 to 2007 (Table 1). In China, specimens were collected from the lower and middle Yangtze River (Hanjiang and Shishou sections), the middle Pearl River (Shaoqing section), and the middle Amur River (Fuyun section). In the United States, specimens were collected from the middle Illinois River and the lower Missouri River within the Mississippi River Basin (MRB). In Hungary, specimens were collected from Szeged and Faks section of the Danube River (DAN). In this study, we pooled samples into five river populations for analysis. A caudal fin clip from each individual was taken and stored in 95% ethanol for genetic analyses.

DNA extraction, PCR amplification, and sequencing

Total genomic DNA from fin tissue was extracted using a proteinase K and phenol-chloroform procedure (Palumbi 1996). Quantity and quality of extracted DNA were estimated on 1% agarose gels stained with ethidium bromide (EB).

The polymerase chain reaction (PCR) was used to amplify a fragment of the mitochondrial COI gene using the primers COI-F (5′-TTAAACCTCTGTCTT CGGGG-3′) and COI-R (5′-CTGGGTGACCAAA GAATCAG-3′). A fragment of the D-loop was amplified using the primers DL-F (5′-ACCCCTGGCTACCCA AAGC-3′) and DL-R (5′-ATCTTAGCATCTTCAGTG-

Table 1 Sampling information and haplotypes found in each river

	River (River basin)	No. of samples	No. of haplotypes	No. of unique haplotypes
China	Yangtze (YZ)	58	24	16
	Pearl (PL)	7	4	3
	Amur (AMU)	56	26	23
Hungary	Danube (DAN)	26	9	5
USA	Mississippi (MRB)	94	20	18

3′). These two pairs of primers were designed from the complete mtDNA sequences of silver carp (Li et al. 2009; GenBank accession NO. NC_01056). PCR was performed using an Eppendorf Thermal Cycler in a reaction mixture of 50 μl containing 25 μl 2xTaq PCR MasterMix, 2 μl primers (0.2 μM each), and 23 μl distilled water. The amplification conditions were detailed as follows: 94°C for 5 min; followed by 30 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 10 min. One percent agarose gel electrophoresis was used to verify successful PCR amplification.

All amplified products were purified using a 3S Spin PCR Product Purification Kit (Biocolor Inc., Shanghai, China) following the supplier's instructions. The purified products were then directly sequenced on an Applied Biosystems ABI 3730 capillary sequencer using the same PCR primers.

Sequence alignment and data analyses

DNA sequences were edited using BioEdit software (Hall 1998), aligned using ClustalW software (Thompson et al. 1994). The revised alignment was 1,262 bp for the COI gene and 732 bp for the D-loop region. The incongruence length difference (ILD) test (Farris et al. 1994) was carried out to test the compatibility of the two mitochondrial segments using the program PAUP version 4.10b (Swofford 2003). The results indicated a congruent phylogenetic signal (p=0.48) for the sequences of these two segments. Therefore, the two datasets were combined for the following analyses.

Population structure and genetic variance were analyzed using Arlequin 3.01 (Schneider et al. 2000). Genetic diversity was analyzed by estimating haplotype diversity (h) and nucleotide diversity (π) using the methods of Tajima (1983) and Nei (1987). The overall genetic differentiation was tested using the pairwise fixation index (F_{ST}) between populations. The analysis of molecular variance (AMOVA) was also calculated using Arlequin 3.01 (Schneider et al. 2000).

The Bayesian approach was employed for inferring the relationship among haplotypes, three million Markov Chain Monte Carlo (MCMC) generations were run with MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003), with a sampling frequency of one in 100 generations. Two independent runs were carried out to allow additional confirmation of the convergence of MCMC runs, with a sampling frequency of one in 100 generations and a burn-in of 10,000 generations. A consensus tree was constructed from the saved trees, with all trees before stabilization was reached being discarded. A median-joining haplotype network (Bandelt et al. 1999) was constructed using Network 4.510 (Fluxus Technology 2008).

Results

Genetic diversity of silver carp populations

A total of 281 variable nucleotide sites and 228 informative sites were observed from 241 specimens in five major rivers (or river systems), resulting in 73 haplotypes (Tables 1 and 2). Sixty-five of these occurred in only one population. The haplotypes YZ-001, YZ-002, YZ-003, YZ-007, YZ-008, YZ-021, YZ-031 and YZ-033 were shared by fish from different rivers (Fig. 1), in which the haplotype YZ-002 was shared by all except the Pearl River populations. Interestingly, no haplotype was shared by all populations of the five river systems.

The haplotype diversity varies from population to population, ranging from 0.7351 (MRB) to 0.9515 (Yangtze River) (Table 2). All three native populations possess higher haplotype diversity as compared with two introduced populations. Similarly, the nucleotide

Table 2 Sequence diversity of silver carp from five rivers or river systems

Population	Variable sites	Parsimonious informative sites	Haplotype diversity	Nucleotide diversity
Yangtze River	93	81	0.9515±0.0141	0.0094±0.0047
Pearl River	28	10	0.8571±0.1023	0.0076±0.0044
Amur River	45	32	0.9253±0.0230	0.0064±0.0033
Danube River	26	21	0.8431±0.0447	0.0056±0.0029
Mississippi River Basin	89	84	0.7351±0.0361	0.0030±0.0016

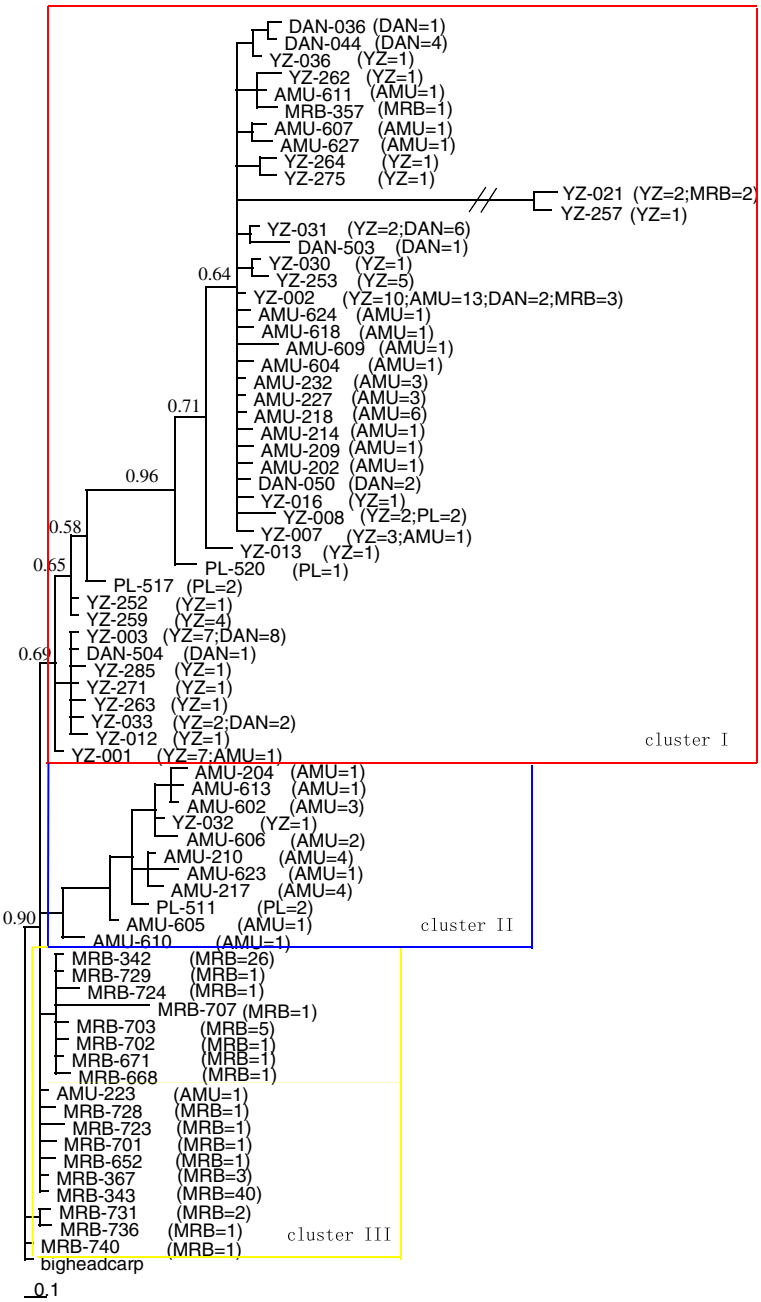

Fig. 1 Bayesian phylogram of silver carp haplotypes. The numbers above the branches denote Bayesian posterior probabilities whereas observed frequencies of haplotypes in corresponding populations are presented in parenthesis after each haplotype. *DAN* Danube River; *MRB* Mississippi River Basin; *YZ* Yangtze River; *PL* Pearl River; *AMU* Amur River

diversity ranged from 0.0030 (MRB) to 0.0094 (Yangtze River), with higher values in native populations than in introduced populations. When comparing samples pooled by countries, the highest haplotype diversity was found in silver carp from China (0.9515) whereas the lowest haplotype diversity was found in fish from the United States (0.7351). Similarly, the highest nucleotide diversity was found in the Yangtze River (0.0094) and the lowest in the MRB (0.0030) (Table 3).

Genetic divergence of silver carp populations

Pairwise F_{ST} analysis showed that between native populations there was significant genetic difference only between the Yangtze and Amur River populations (F_{ST}=0.0716; p<0.01). The number of shared haplotypes is three between YZ and AMU, one between YZ and PL and zero between PL and AMU. The genetic difference between the two introduced populations is relatively high (F_{ST}=0.4782; p<0.01), which is the second highest value among all the pairwise comparisons. The highest F_{ST} was found to be 0.4951 between the MRB and the Amur River. The extent of genetic variation between native and introduced populations was different between the MRB and Danube River populations. There was significant genetic differentiation between the MRB and each of the native populations, with F_{ST} ranging from 0.3258 to 0.4951. In contrast, the Danube River population had no genetic differentiation as compared with the Yangtze population.

The analyses of molecular variance revealed significant genetic difference between five populations (P< 0.001), in which 23.95% variance was contributed by the difference among populations. Genetic difference was also found between native versus introduced groups (F_{ST}=0.2177, P<0.05). Surprisingly, there was no genetic difference among populations pooled by countries. This may be caused by the unbalanced number of populations from different countries (three in China and one in the US) and the varied number of samples (Table 4).

Table 3 Pairwise F_{ST} and the significance between silver carp populations

	Yangtze	Pearl	Amur	Danube
Yangtze				
Pearl	0.0059^{NS}			
Amur	0.0716 **	0.0637 ^{NS}		
Danube	0.0142 ^{NS}	0.1178*	0.1001 *	
MRB	0.3258**	0.3950 **	0.4951 **	0.4782**

*P<0.05; **P<0.01; *NS* not significant

Evolutionary relationship among silver carp populations

The relationship of haplotypes and the observed haplotype frequencies are shown in Fig. 1. Three major haplotype clusters were observed, consisting of 44 haplotypes (cluster I), 11 haplotypes (cluster II) and 18 haplotypes (cluster III). Cluster I includes silver carp from all the five river systems. Interestingly, all shared haplotypes appeared in this cluster. Cluster II consists of samples from China, but more are from the Amur River. In cluster III, all fish except one AMU sample are from the MRB.

The haplotype network shows strong connection among the three native populations, but there was no obvious connection between the Pearl and Amur river populations (Fig. 2). Most MRB haplotypes are interconnected and linked with the YZ haplotypes through the haplotype AMU-223. On the contrary, the DAN haplotypes are less interconnected, with three ending haplotypes apart from each other.

Discussion

Origins of silver carp and its introduced populations

Our study provides strong evidence to support the hypothesis that silver carp originate from the Yangtze River. First, all eight haplotypes found in the Yangtze River population were shared with other populations. The haplotype YZ-002 was found in all populations except the Pearl River population (which had a low sample size and thus the haplotype may have been missed) and is likely to be an ancestral haplotype. Secondly, the "native" Amur and Pearl River populations shared haplotypes with the Yangtze River and not with each other. Thirdly, because of the Yangtze River's size, central location, geological and fossil history (Li and Fang 1990), and the historical abundance of the four domesticated Chinese major carps (silver carp, bighead carp, grass carp and black carp), it has long been believed that the Yangtze River is the evolutionary source of these fishes.

Table 4 Percentage of variation, fixation indices from AMOVA and SAMOVA with the largest F_{sT} value of silver carp

Category	Source of variation	Df	Percentage of variation	Fixation indices (Fst)	*P*-value
Among five populations	Among populations	4	23.95.	0.2395	<0.001
	Within populations	236	76.05		
Among three countries	Among countries	2	25.83	0.2583	0.2063
	Within countries	2	6.98	0.0941	<0.001
	Within populations	236	67.19	0.3281	<0.001
Between native and introduced	Between groups	1	21.77	0.2177	0.0098
	Within groups	3	9.57	0.1223	<0.001
	Within populations	236	68.67	0.3133	<0.001

The MRB population may have ancestry at least from the Amur or the Yangtze River. First, the haplotype YZ-021 was shared between YZ and MRB populations (Figs. 1 and 2). Second, the haplotype AMU-223 was clustered with other haplotypes unique to MRB haplotypes. There are two known importations of silver carp to the United States, one from Taiwan, probably in 1971; and a later importation from Yugoslavia (Kolar et al. 2007). The original importation was widely distributed soon after importation, and there is no further information on the disposition of the Yugoslavian importation. In any case, silver carp are not indigenous to Taiwan or Yugoslavia, and there is no clear record of the original sources. Chen (1990) stated that silver carp imported to Taiwan came from Mainland China or Japan; Japan is also not within the native range of silver carp.

The Danube River population is likely to originate from the Yangtze River, the Amur River, or both (Fig. 1). The Danube River drains much of Europe, and there are many known importations to the many countries within the Danube basin (Kolar et al. 2007), many from other countries within the basin, but known importations from outside the basin came directly from China or from Russia. The Amur River borders Russia, therefore silver carp from Russia may have originated there, but may also have been imported from other Chinese sources.

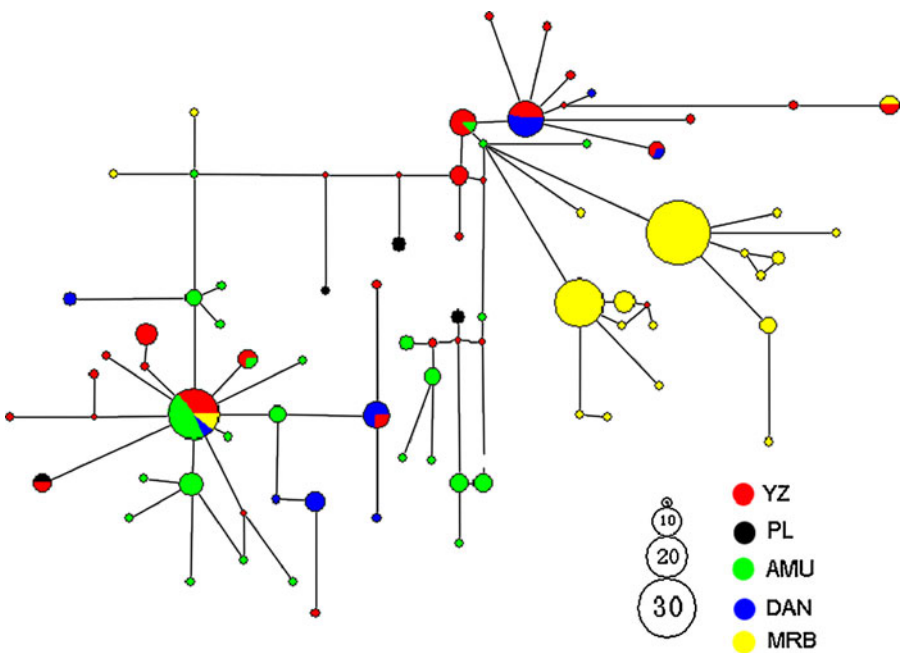

Fig. 2 Haplotype network showing haplotype connections and distribution. *Colors* correspond to different populations. *Circle size* denotes grades by 10 of pooled number of each haplotype

Genetic diversity of native and introduced populations

Compared with native populations, introduced populations had lower haplotype diversity and nucleotide diversity, which would logically result from founder effects caused by small introduced populations. The total number of fish imported to the Danube and MRB basins is unknown but was probably low. Also, silver carp are somewhat difficult to spawn in aquaculture, but when artificial spawning is successful, very large numbers of young fish can be obtained from a single female. For example, the absolute fecundity of 6-year-old and 7321±894 g body weight silver carp females was $8.63 \pm 1.55 \times 10^5$ eggs (Li 1998). This would further increase the genetic bottleneck, because aquaculturists have little incentive to spawn many broodstock. The MRB population had substantially lower haplotype and nucleotide diversity than any other population, including the also-introduced Danube population, which makes sense in light of the importation and culture history. Silver carp were imported to the Danube basin in the early sixties from China and from Russia, but the total number of importations is unknown. They were (and continue to be) cultured extensively in many countries of the basin (Kolar et al. 2007), thus opportunities for escape have been plentiful. Also, in some cases, silver carp were intentionally stocked to the wild in the Danube basin (Antalfi and Tölg 1972). In contrast, in North America, silver carp were imported approximately 10 years later, from two separate sources, neither from the native range of the fish (Kolar et al. 2007). Silver carp have not been widely used in aquaculture in the United States, so opportunities for their escape were few. Only fish from the Taiwan importation are known to have been widely distributed for aquaculture and research in North America (Kolar et al. 2007). Furthermore, there is no evidence that silver carp have ever been intentionally stocked into the wild in North America. These factors should further increase the genetic bottleneck in North America, and are likely responsible for the reduced haplotype and nucleotide diversity. Nevertheless, the MRB population had several unique haplotypes and was significantly differentiated from other populations. This occurred in less than 35 years, only a few generations of silver carp, suggesting that unique ecological or environmental characteristics are promoting divergence of the MRB population.

It should be noted, however, that the low nucleotide diversity of MRB silver carp stands in contrast to that of bighead carp, which was first imported to North America together with the first importation of silver carp. In a similar study with bighead carp (Li et al. 2010) haplotype diversity was lowest in the MRB, but nucleotide diversity was substantially higher than in populations from the other four river systems. Bighead carp and silver carp were imported together in the same shipments from Taiwan and Yugoslavia, and we know of one additional importation of bighead carp, from Israel (Kolar et al. 2007). Unlike silver carp, bighead carp have been and continue to be somewhat widely cultured in the MRB, therefore some of the high nucleotide diversity in bighead carp might be related to aquaculture activities and continued escape of bighead carp. However, bighead carp and silver carp both are much more widely cultured in China and Eastern Europe than in North America, so it is difficult to ascribe the increased nucleotide diversity entirely to aquaculture activities. Perhaps evolutionary pressures in the MRB that acted differently on bighead carp and silver carp are responsible for this difference.

Genetic variation of introduced populations

Silver carp were introduced into the United States approximately 30 years ago and to Hungary approximately 40 years ago (Kolar et al. 2007). Due to the strong founder effect, it is not surprising that all but two of the haplotypes we identified in silver carp from their native ranges were not present in the MRB, and that overall haplotype diversity was very low in the MRB. However, we found significant genetic differentiation between the MRB and all three native populations, and we found 18 haplotypes that were unique to the MRB. Silver carp are thought to become reproductive in 2 to 4 years, thus these haplotypes apparently occurred within only 8–15 generations, unless those haplotypes are present at low frequency in the native range and also present in the small number of imported and spawned broodstock. It is unlikely that these haplotypes were introduced through culture activities, because silver carp have been little cultured in the United States, and conversely are much cultured in the Danube basin and in China. Additionally, the environmental settings are quite different in native and introduced river systems. For example, although both are among the world's largest rivers, the Mis-

sissippi River flows southwards from Minnesota to the Gulf of Mexico, whereas the Yangtze River flows eastwards to the East China Sea. Furthermore, the extensive network of connected floodplain lakes that characterize the lower Yangtze basin, and which is considered the primary natural living and growing habitat of silver carp (Kolar et al. 2007), is not emulated in North America. Thus it is plausible that natural selection in a novel environment like MRB might have occurred in favor of genetic differentiation of the introduced silver carp. Rapid evolution has often been identified in introduced populations (Cox 2004), and it may be occurring here.

In contrast, in the Danube River, where the fish have a longer history and a much greater history of aquaculture, there was no significant differentiation from the Yangtze population. In addition, we found only five unique haplotypes, and four haplotypes shared with the Yangtze River. The reasons for this discrepancy are unclear, but may be because of a greater number of importations to the region (Kolar et al. 2007) and a correspondingly lesser founder effect, or it may be because environmental evolutionary pressures are not as strong. Also, the number of samples from the Danube River was not as high as from the MRB, and haplotypes of moderate or low frequency may have been missed.

Silver carp have been expanding their range northward in North America and are poised to invade the Laurentian Great Lakes. There is much concern that they will become established there to the detriment of highly valued fisheries. We found that silver carp were most closely related to both the Yangtze River population in central China and to the Amur River population on the northern border of China, and not closely related to the Pearl River population in southern China. The relationship with these more northern populations may be important in estimating the risk of silver carp invasion of the Great Lakes.

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