Native bighead carp *Hypophthalmichthys nobilis* and silver carp *Hypophthalmichthys molitrix* populations in the Pearl River are threatened by Yangtze River introductions as revealed by mitochondrial DNA

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Abstract

Bighead carp *Hypophthalmichthys nobilis* and silver carp *Hypophthalmichthys molitrix* have been two economically important aquaculture species in China for centuries. In the past decades, bighead and silver carp have been introduced from Yangtze River to many river systems in China, including Pearl River, in annual large-scale stocking activities to enhance wild fisheries. However, few studies have assessed the ecological or genetic impacts of such introductions on native conspecific fish populations. We obtained a mtDNA D-loop segment of 978 bp from 213 bighead carp samples from nine populations and a 975 bp segment from 204 silver carp samples from ten populations to evaluate genetic diversity and population integrity. Results from a haplotype network analysis found that most Pearl River haplotypes clustered with haplotypes of Yangtze River origin and only a small proportion were distinct, suggesting that native Pearl River bighead and silver carp populations are both currently dominated by genetic material from Yangtze River. Genetic diversity of Pearl River populations is high in both species because of this inter-population gene flow, but native Pearl River diversity is low. We propose that, to preserve the native genetic diversity, stocking of non-native fingerlings should cease immediately and native Pearl River bighead and silver carp fish farms should be established. This research demonstrates the danger to native biodiversity across China of the substantial ongoing stock enhancement activities without prior genetic assessment.

KEYWORDS Bighead carp, Silver carp, Pearl River, Genetic diversity, Domestic

57 introduction, Stock enhancement

1 | INTRODUCTION

Bighead carp *Hypophthalmichthys nobilis* (Valenciennes 1844) and silver carp *Hypophthalmichthys molitrix* (Richardson 1845), belonging to Cypriniformes, Cyprinidae (Chen, 1998), are two major commercial species in China, which now rank second and third, respectively, in Chinese freshwater aquaculture production (MOA, 2018) and second and fifth, respectively, in global aquaculture production (FAO, 2018). They are native to China, with a natural range extending from Pearl River in the south to Amur River in the north (Li *et al*., 1989). Since the 1950s, bighead and silver carp have been introduced or spread into more than 88 countries around the world, primarily for use in aquaculture, but also for plankton control and fisheries enhancement (Kolar *et al*., 2007; Li *et al*., 2010; Li *et al*., 2011). In some of these places, the carps have had serious negative ecological effects and are considered as invasive species (Irons *et al*., 2007). For example, in North America bighead and silver carp have spread throughout the Mississippi River Basin and increased in population size and biomass, resulting in negative environmental consequences and a pressing issue for fisheries scientists (Sandra, 2016).

Conversely, since the 1960s alarming declines of both carp species have been observed in the Yangtze River and Pearl River due to the construction of dams, pollution, and overfishing (Li *et al*., 2008; Mao *et al*., 2010). For example, the Yangtze River yielded about 30 billions of fry for the four Chinese carps annually in the early 1960s, which subsequently declined to two billions in 1980 and one billion in 2010 (Mao *et al*., 2010). Therefore, the two carp species included here are currently

80 classified as near threatened in China (Zhao, 2011; Huckstorf, 2012). Although both
81 species in these two rivers are decreasing quickly, the situation differs between
82 Yangtze River and Pearl River. Historically, the Yangtze River was the main
83 production area of the four major Chinese carp species in China, followed by Pearl
84 River and Amur River (Li, 1990). The fish fry used for aquaculture in these rivers
85 were mainly captured from wild populations until the success of artificial propagation
86 approaches in 1958. Since then, only few wild individuals were collected for breeding
87 every year. More recently, the construction of the Three Gorges Dam caused concern
88 for its huge negative impact on the ecology and fisheries of the Yangtze River (Zhang
89 *et al*., 2012), which led to the establishment of four national breeding farms in the part
90 of the river below the dam to restore wild fisheries. These subsequently served as the
91 main origin of carp fry across China, because Yangtze River populations have
92 superior desirable traits for aquaculture, such as a bigger body size at maturity and a
93 faster growth rate (Li, 1990).

94 The Pearl River basin is considered the "lifeline" of terrestrial water resources in
95 China, which comprises three main tributaries: Xijiang River (usually represents the
96 Pearl River because it is the longest in the three tributaries), Beijiang River (the
97 second longest), and Dongjiang River. It has a different evolutionary history from
98 Yangtze River (Li, 1981; Wang, 1994). For example, bighead and silver carp, which
99 originated from the Yangtze-Yellow eastern plain of China in the Pliocene about 3.5
100 million years ago, arrived at the Pearl River through the Yangtze River and the
101 Qiantang River ca 110,000 years ago during the Pleistocene (Li & Fang, 1990), in the

course making the native carp populations evolutionarily distinct (Li *et al*., 2010; Li *et al*., 2011). Nonetheless because of the perceived superiority of Yangtze stock, bighead and silver carp stock enhancements have been carried out in Pearl River since 2001 (Mao *et al*., 2010). These activities were performed under unscientific and varying rules in the basin, without knowing the genetic diversity and parental origin of the released fingerlings, but which were believed to have originated from the Yangtze River (Wu *et al*., 2016). Research on bighead carp collected from Yangtze River breeding and wild populations and a Pearl River fish farm population using 17 microsatellite loci suggested that the Pearl River-cultured population genetically clustered with the Yangtze River wild population (Zhu *et al*., 2018), providing evidence that introductions have become established. For these reasons, the native Pearl River carp populations are likely in danger of being replaced by introduced carp populations from the Yangtze River. With the increasing introductions from Yangtze River and large-scale, yearly stock enhancement of these introduced carps into Pearl River, a detailed survey focusing on the genetic diversity and status of bighead and silver carp are critically important for the conservation of native Pearl River populations.

Although there are many published studies that addressed the genetic diversity of bighead and silver carp, most research in China has mainly focused on assessing fisheries and comparisons of genetic diversity among different populations of a species within the Yangtze River (Li, 1990; Li & Wang, 1990; Li *et al*., 1998; Zhang *et al*., 1999; Geng *et al*., 2006; Shan *et al*., 2006; Wang *et al*., 2008; Li *et al*., 2010;

Zhang *et al*., 2013), providing a good reference for comparison of genetic diversity of potential introduced Yangtze River populations and native Pearl River populations. On the other hand, studies on the genetic diversity of bighead and silver carp in Pearl River (Li *et al*., 2010; Liu *et al*., 2010; Li *et al*., 2011; Wu *et al*., 2016) showed low genetic diversity and proposed stock enhancement activities to recover wild fisheries. However, the limitation of their sampling size constrained our understanding of the real genetic diversity of carps in the Pearl River. In addition, the genetic diversity and status of released carp individuals and their ecological impact on local populations remain unknown. The fact that native populations can suffer from introgressive hybridization with alien stocks of domestic origin has been well demonstrated, for example in Atlantic brown trout (Caputo *et al*., 2004; Lorenzoni *et al*., 2006). In that example, the Italian native trout showed various degrees of hybridization of two mitochondrial lineages, with some populations totally replaced by non-native trout (Splendiani *et al*., 2016), and a similar issue may occur in bighead and silver carp in China.

In the present study, we aimed to (i) assess if colonization of bighead carp and silver carp from Yangtze River to Pearl River is evident and, (ii) if so, identify the extent to which the genetic material of Yangtze River bighead and silver carp have been established in the Pearl River populations, and (iii) its impact on the genetic diversity of these two species in Pearl River. The most important tributaries of Pearl River were sampled in order to evaluate the genetic diversity of native, non-stocked populations. The partial mitochondrial DNA control region (D-loop) was chosen

146 rather than microsatellites because native populations between rivers are known to be
147 distinct at mtDNA marker (Li *et al*., 2010; Li *et al*., 2011) and this marker is suitable
148 for tracking the long evolutionary history of animal species (Moritz *et al*., 1987), has
149 an estimated mutation rate and predictable evolution, and is comparable across studies
150 and species, unlike other markers such as for example microsatellites (Noor *et al*.,
151 2001). Based on our findings, possible conservation strategies of the remaining native
152 Pearl River populations of bighead and silver carp are proposed.
153

154 **2 | MATERIALS AND METHODS**

155 **2.1 | Sampling and DNA extraction**

156 The care and use of experimental animals complied with China animal welfare
157 laws, guidelines and policies as approved by South China Normal University (permit
158 reference number No.201303048). Fishes were collected for conservation purposes
159 and to contribute to the fisheries knowledge of these species. All sampled fish were
160 fatally anesthetized with MS-222 (Sigma).

161 We surveyed nine wild populations of co-existing bighead and silver carp in Pearl
162 River basin between July 2013 and December 2015 by using gill nets. Individuals
163 were selected according to their body size and total length. Populations were selected
164 based on their natural distribution in the Pearl River (Chen, 1998; Pan *et al*., 1990).
165 The age of individuals was estimated according to length-age relationships. All
166 individuals were juvenile (between one to three years old, standard length ranges from
167 26 to 44 cm; at this stage they can be easily identified). The specimens were identified

based on the current literature (Chen, 1998; Wang, 1994). In addition, fry of silver carp were sampled from Xijiang River of Pearl River in July 2017 and raised for four months in the laboratory and used for molecular analysis. A total of 213 individuals of bighead carp were collected from Qingyuan (BJ, N=24), Longchuan (DJ, N=30), Fengkai (XJYY, N=17) and Zhaoqing (XJ, N=23) in Guangdong Province, from Shaoping (GJ, N=12), Laibin (HSH, N=30), Liuzhou (LJ, N=23), Pingguo (YJ, N=26) and Guiping (YUJ, N=9) in Guangxi Province, and from Luoping (NPJ, N=36) in Yunnan Province. A total of 187 individuals of adult silver carp were collected from Qingyuan (BJ, N=20), Longchuan (DJ, N=30) and Zhaoqing (XJ, N=16) in Guangdong Province, from Shaoping (GJ, N=5), Laibin (HSH, N=30), Liuzhou (LJ, N=18), Pingguo (YJ, N=13) and Guiping (YUJ, N=21) in Guangxi Province, and from Luoping (NPJ, N=34) in Yunnan Province. Seven sequences of bighead carp and 64 sequences of silver carp were downloaded from the NCBI database, which were originally sampled from Jianli in Hubei province beside the Yangtze River (Hao *et al*., 2013; GenBank Accession Numbers for bighead carp: KC292939-KC292945; GenBank Accession Numbers for silver carp: KC292923-KC292927, KC292929-KC292934, KF384046-KF384088, KF384089-KF384096) (Table 1, Figure 1).

For molecular analyses, a piece of muscle tissue was obtained from each individual and preserved in 95% ethanol or frozen. DNA was extracted using standard phenol-chloroform extraction protocols (Sambrook *et al*., 1989), and the partial D-loop sequence was amplified using polymerase chain reaction (PCR) with the

190 primers DL1 (5'-ACCCCTGGCTCCCAAAGC-3') and DH2
191 (5'-ATCTTAGCATCTTCAGTG-3') (Liu *et al*., 2002). Each 25 μL PCR reaction
192 contained 1.0 μL template DNA, 13 μL 2×*Taq* PCR MasterMix (0.1U *Taq* DNA
193 Polymerase/ml, 500 mM per dNTP, 50 mM Tris-HCl (pH 8.7), 20 mM KCl, 4 mM
194 $MgCl_2$), 10 ng of each primer, and 9 μL ddH_2O. PCR amplification was conducted in
195 a thermal cycler (Eppendorf Master cycler) including a negative control using the
196 following conditions: one cycle of denaturation at 94°C for 2 min; 35 cycles of
197 denaturation at 94°C for 45 s, annealing at 61°C for 45 s, and extension at 72°C for
198 1.2 min; followed by extension at 72°C for 7 min and storage at 4°C. PCR products
199 were purified by electrophoresis in 1.0% agarose gel using a 1×TAE buffer. The gel
200 was stained with ethidium bromide, and the DNA band was cut and eluted using the
201 Agarose Gel Purification Kit (QIAGEN, Valencia, CA, USA). The PCR products
202 were then subjected to cycle sequencing reactions prescribed by Sangon Biotech
203 (Shanghai) Co., Ltd using the DL1 primer. Sequences were obtained using an ABI
204 PRISM 3730XL sequencer with the BigDye Terminator kit (Applied Biosystems). All
205 sequences are deposited in GenBank under accessions MN641494-MN641562 for
206 bighead carp and MN641563-MN641680 for silver carp.
207
208 **2.2 | Data analysis**
209 Nucleotide sequences were aligned using Clustal X v2.1 (Thompson *et al*., 1997).
210 The output files were checked by eye with MEGA v7.0 (Kumar *et al*., 2016) and
211 haplotypes were identified with the default settings in DnaSP 5.10 software (Librado
212 & Rozas, 2009). Genetic distances between groups were calculated using MEGA v7.0

213 with a Kimura two-parameter (K2P) genetic distance model. Genetic diversity was
214 quantified based on haplotype diversity (*h*) and nucleotide diversity (π) (Nei & Tajima,
215 1981) using DnaSP 5.10 software. The differences between populations were assessed
216 with pairwise genetic distance and genetic differentiation index (*Fst*) values
217 calculated in DnaSP 5.10 software. Haplotype networks were constructed using the
218 median-joining network method (Bandelt *et al*., 1999) in Network 4.6.1.0. software.
219 Demographic history was inferred using a mismatch analysis (Rogers & Harpending,
220 1992). The Tajima's *D* (Tajima, 1989), Fu's *Fs* (Fu, 1997), Harpending's raggedness
221 index, and the sum-of-squared deviations statistics (SSD) were calculated in Arlequin
222 3.5.2.2 (Excoffier & Lischer, 2010).
223
224 **3 | RESULTS**
225 **3.1 | Genetic diversity of bighead carp**
226 The alignment of all D-loop sequences revealed 188 polymorphic positions, of
227 which 134 positions were parsimony informative. A total of 69 haplotypes were
228 identified from the 213 samples, with 68 haplotypes in Pearl River and six haplotypes
229 in the Yangtze River, five of which were shared with Pearl River (83.3%) (Table 2;
230 Supporting Information Figure S1). The overall haplotype diversity of Pearl River
231 populations combined was 0.936. The within-population haplotype diversity values
232 were high (>0.85) in all populations (Table 2). The overall nucleotide diversity of
233 Pearl River populations combined was 0.00565. The highest nucleotide diversity was
234 observed in Xijiang River population (XJ; π=0.00727), while the lowest was observed

235 in the Yujiang River population (YUJ; π=0.00282). Populations BJ, DJ, and LJ also
236 had high nucleotide diversity (Table 2). Pearl River and Yangtze River populations
237 had similar levels of genetic diversity (h=0.936, π=0.00569 vs h=0.952, π=0.00526).
238
239 **3.2 | Genetic diversity of silver carp**
240 222 polymorphic positions were detected in all D-loop sequences with 172
241 parsimony informative positions. 118 haplotypes were obtained of the 204 silver carp
242 samples. 98 haplotypes were from the Pearl River and 25 haplotypes were from the
243 Yangtze River. 20 of Yangtze River haplotypes were shared with the Pearl River
244 (80.0%) (Table 3; Supporting Information Figure S2). The overall haplotype diversity
245 of Pearl River populations was 0.963 (range: 0.446-1). The within-population h values
246 were high in all populations except population DJ (Table 3). The nucleotide diversity
247 of all Pearl River populations combined was 0.02174. The highest nucleotide
248 diversity was observed in Beijiang River population (BJ; π=0.03926), while the
249 lowest was observed in Dongjiang River population (DJ; π=0.00619) (Table 3).
250 Overall the genetic diversity of silver carp was higher in Pearl River (h=0.968,
251 π=0.02474), than in Yangtze River (h=0.897, π=0.00864), though Pearl River also
252 had more individuals and more localities sampled.
253
254 **3.3 | Haplotype network analysis**
255 **3.3.1 | Bighead carp**
256 The haplotype network analysis identified two clades in bighead carp, with six
257 mutation steps between them (Figure 2). Clade I contained 65 haplotypes found in

Yangtze and Pearl River and Clade II comprised only four haplotypes (5.8% of total haplotypes), which are found exclusively in Pearl River (Table 2). Despite haplotype relationships between populations, the network analysis also showed that the dominant haplotypes (e.g., H3, H11, H19) were shared between Yangtze River and Pearl River samples (Figure 2). Besides, five out of six of Yangtze River haplotypes were shared with Pearl River generally (Figure 2) and found in many individual populations (e.g., population BJ, DJ and XJ) (Supporting Information Figure S1). For example, population XJ had the H3 haplotype from Yangtze River and a H55 haplotype from Clade II, which is otherwise native Pearl River. Most individuals in population DJ had the same haplotypes as Yangtze River samples, especially the H19 haplotype. Populations BJ, DJ, and LJ had one or two haplotypes grouped in Clade II (Supporting Information Figure S1).

3.3.1 | Silver carp

In the haplotype network analyses, 118 mtDNA haplotypes fell into two divergent lineages (I and II). The number of haplotypes clustered in Lineage II was clearly lower than that in Lineage I (17 vs 101) (Figure 3, Table 3). Haplotypes from lineage II formed two sublineages with ten mutation steps between them. The number of mutations between two lineages in silver carp was considerable, at 57 steps. Seventeen silver carp samples from Xijiang River (XJYY) contained both lineages of this species (Table 2; Figure 3; Supporting Information Figure S2). The haplotype H14 and H22 were the most frequent in sublineage A and B, respectively. All of these

dominant haplotypes in Lineage I were shared by Yangtze and Pearl River populations (Figure 3). What is more, they were observed in most Pearl River populations (e.g. populations BJ and DJ) (Supporting Information Figure S2). However, population XJ did not share any frequent haplotypes with Lineage I, with some unique haplotypes clustered with Lineage II. Interestingly, the most frequent haplotype in population DJ was the H22 haplotype, which did not occur in other Pearl River populations. Populations BJ, HSH, XJ, and XJYY had one or two haplotypes grouped in Lineage II (Supporting Information Figure S2). Two populations (XJ and XJYY) from the Xijiang River (downstream of Pearl River) had a similar proportion of Lineage I and II haplotypes (Table 3).

3.4 | Genetic differentiation

We calculated the genetic distance and genetic differentiation index (*Fst*) between lineages or clades identified based on haplotype analysis. Results showed that the genetic distance between the two lineages in silver carp was larger (Table 5) than that between the two clades of bighead carp (Table 4).

The pairwise K2P genetic distances revealed low genetic differentiation among all populations of bighead carp, ranging from 0.004 to 0.007 (Supporting Information Table S1).

K2P genetic distances among most populations of silver carp were high, from 0.009 to 0.038 across sites (Supporting Information Table S2). Genetic distances between silver carp populations with high nucleotide diversity within Pearl River (e.g.,

302 BJ and LJ) was larger than other pairs (e.g., GJ and DJ) (Supporting Information
303 Table S2).
304
305 **3.5 | Demographic history**
306 Fu's *Fs* and Tajima's *D* statistics for bighead and silver carp were significantly
307 negative except for Tajima's *D* for Clade II of bighead carp and Lineage I of silver
308 carp (Supporting Information Table S3). Apart from Clade II of bighead carp, both
309 species showed a multimodal distribution in the mismatch analyses (Supporting
310 Information Figure S3). The Harpending's raggedness index was significant for Clade
311 I of bighead carp and the SSD was significant for Lineage II of silver carp
312 (Supporting Information Table S3).
313
314 **4 | DISCUSSION**
315 **4.1 | Evidence for colonization from Yangtze River**
316 In this study, we aimed to assess if and to what extent colonization of bighead
317 and silver carp from Yangtze River has taken place. Similar to other fish species, such
318 as in the brown trout species complex (Splendiani *et al*., 2016), we clearly
319 demonstrated mitochondrial gene flow from the Yangtze River into the Pearl River
320 (Figure 2, 3). 'Gene flow' in this study could mean introgression, hybridization, or
321 replacement of one lineage by another by colonization or anthropogenic introduction.
322 We cannot exclude the possibility of migration but think it is unlikely because of the
323 biogeographic barriers between Pearl and Yangtze River.
324 For bighead carp, the haplotype network analysis identified two clades: clade I

contained 65 haplotypes from Yangtze and Pearl River and Clade II comprised only four Pearl River haplotypes (Table 2), suggestive of the close but distinct phylogenetic relationship of Yangtze and Pearl River populations. Genetic distance between these two clades was equivalent to the inter-population level (Table 4), which suggests that Clade I consists of original Yangtze River haplotypes and Clade II is comprised of native Pearl River fish. As shown, most haplotypes of Yangtze samples were shared with Pearl River samples (Figure 2), indicating these haplotypes were likely introduced from Yangtze River. Here, we argue that colonization from the Yangtze River is the most likely cause for the observed pattern rather than shared ancestral polymorphism for the following reasons: (1) according to Zhu *et al*. (2018), their findings showed that samples from fish farms producing fish fry used for stock enhancement activities were in fact introduced from the Yangtze River; (2) no private haplotypes of Yangtze River were detected in Yangtze River samples while there are some in Pearl River samples (Clade II haplotypes). Although the sample size of the Yangtze River population of bighead carp was small, it is much larger than the Yangtze River population of silver carp and no unique haplotypes of the Yangtze River population were found either; (3) in nature, bighead carp spawns in rivers; their offspring migrate to lakes and stays there until maturity (Chen, 1998; Pan e*t al*., 1990). Eggs of bighead carp can only hatch under sufficient accumulated temperature, which means they cannot survive in fragmented rivers that resulted from dam construction (Li & Fang, 1990; Mao *et al*., 2010). Populations present in these rivers (e.g., DJ and upstream population, NPJ) shared most of their haplotypes with Yangtze River

347 samples (Supporting Information Figure S1, S2), providing a strong evidence of
348 introduction. However, we cannot reject the possibility that there is some shared
349 ancestral polymorphism between these two river systems. A more detailed survey of
350 all available populations in Yangtze and Pearl River using both nuclear and
351 mitochondrial markers would be valuable. Given these patterns of bighead carp, and
352 that three dominant haplotypes of Yangtze River are shared with all Pearl River
353 populations (Supporting Information Figure S1), our findings suggest that all the
354 present Pearl River populations have been invaded by fish of Yangtze River origin.
355 Similarly, for silver carp, the haplotype network analysis showed most
356 haplotypes of Pearl River clustered in Lineages I with Yangtze River haplotypes
357 (Figure 3). Given that the number of mutations (57 steps) and genetic differentiation
358 between two lineages in silver carp in Pearl River was considerable (Table 5; Figure
359 3), it is unlikely that the two lineages evolved in the Pearl River because there are no
360 geographic barriers in the river system and no similar results have been found in other
361 fishes at this genetic marker in the basin (Han *et al*., 2010; Yang *et al*., 2016; Li *et al*.,
362 2018). Thus, a reasonable explanation for the results is that Lineage I consists of fish
363 of the original Yangtze River population while Lineage II is formed by pure Pearl
364 River fish.
365 According to Li & Fang (1990), the divergence time of the Yangtze and Pearl
366 River populations of silver carp was about 110,000 years ago during the Pleistocene,
367 which could be old enough for the evolution of the observed divergence. Two
368 sublineages detected in Lineage I (native Yangtze River population) (Figure 3;

Supporting Information Figure S2) were consistent with a previous study on native Yangtze River populations (Sha *et al*., 2018). Apart from mtDNA evidence given by this study, eight microsatellite markers used on Pearl River (sampled from Nanpangjiang River, upper Pearl River) and Yangtze River (sampled from middle location of Yangtze River) wild populations as well as Pearl River culture stocks (sampled from a fish farm in lower Pearl River) showed similar genetic structure and high gene flow among them (unpublished data), suggestive of the same pattern as seen in bighead carp showing that these Pearl River fish farm populations originated from Yangtze River and therefore facilitate the genetic pollution by Yangtze River silver carp.

As in bighead carp, we cannot exclude the possibility that there is shared ancestral polymorphism in silver carp between these two river systems, but we consider it unlikely. Given that the 17 silver carp fry (population XJYY) sampled from Xijiang River consisted of two divergent lineages (putatively native Yangtze and Pearl River populations, respectively) (Figure 3), we can conclude that the Yangtze River lineage of silver carp has founded a natural population in this main stream of Pearl River. What is more, haplotypes in Lineage I were shared by Yangtze and all Pearl River populations (Figure 3; Supporting Information Figure S2), demonstrating that mitochondrial gene flow has affected sampled populations.

The Fu's *Fs* and Tajima's *D* statistics for bighead and silver carp were not consistent with the mismatch distribution analysis. For example, for Clade II the mismatch distribution of bighead carp was unimodal while the Fu's *Fs* and Tajima's *D*

391 statistics were not significant. However, this non-significant result could be because
392 of the small sample size of Clade II. What is more, combined with the low nucleotide
393 diversity and high haplotype diversity patterns of these two species, the significantly
394 negative values for Fu's *Fs* and Tajima's *D* of all other clades indicate these two
395 species likely experienced a bottleneck followed by a rapid population expansion
396 event. These results could be related to sea level and climate change during the
397 Pleistocene, and warrant further investigation.

398 In sum, we concluded that mitochondrial gene flow from the Yangtze River into
399 the Pearl River has occurred extensively. This conclusion was also strengthened by
400 knowledge of the authors that bighead and silver carp were introduced at the same
401 time in aquaculture in China. Importantly, Clade I and II identified in bighead carp
402 and Lineages I and II identified in silver carp corresponded with native Yangtze and
403 Pearl River populations, respectively.

404

405 **4.2 | Impacts of colonization on genetic diversity**

406 As discussed above, mitochondrial gene flow between Yangtze River and Pearl
407 River was observed across all sampled populations. Hence, it is reasonable that the
408 high genetic diversity of bighead and silver carp (Table 2, 3; Supporting Information
409 Figure S1) was found due to mitochondrial gene flow.

410 In bighead carp, the haplotype diversity was high (h=0.936,) while the
411 nucleotide diversity was relatively low (π=0.00569) (Table 2), indicating that
412 populations in the Pearl River may have experienced a population expansion (Grant
413 & Bowen, 1998), consistent with recent population growth after the introduction of

414 Yangtze River individuals. This agrees with previous published results (Li *et al*., 2010)
415 showing that the genetic diversity of the Pearl River population was slightly higher
416 than that of Yangtze River. The total genetic diversity of all sampled populations was
417 slightly higher than that of population CJ (Table 2) and introduced populations from
418 the Yangtze River (Wu *et al*., 2016), supporting mitochondrial gene flow as the cause
419 for increased genetic diversity. Our results are also consistent with a previous study
420 about genetic diversity of bighead carp in Hongshuihe River (upstream of the Pearl
421 River) (Wu *et al.*, 2016). For individual populations, impacts of mitochondrial gene
422 flow from Yangtze River into Pearl River were also found. For example, population
423 XJ has the highest nucleotide diversity and its haplotypes grouped in both Clade I and
424 II of bighead carp, indicating the contact of divergent clades in this population – and
425 other populations such as BJ, DJ, and LJ - resulted in the increased genetic diversity.
426 In silver carp, the genetic diversity was higher in Pearl River than in Yangtze
427 River, also suggesting evidence of colonization from Yangtze River. When focusing
428 on the genetic diversity of populations, several populations (population XJ, XJYY, BJ,
429 etc.) have values exceeding the average. Based on the haplotype network analysis
430 (Supporting Information Figure S2), we can see that all these populations have
431 haplotypes of both Lineage I and Lineage II. Native Pearl River populations without
432 non-native mitochondrial haplotypes may still exist, and future research using both
433 mtDNA and nuclear molecular makers will improve our understanding of where the
434 native carp populations are in the Pearl River system.
435 Interestingly, the population DJ which was sampled from the Dongjiang River,

436 one main tributary of Pearl River, showed a low haplotype diversity (h=0.446). Since
437 the natural environments in the Dongjiang River could no longer meet the
438 requirements for the breeding of silver carp due to the cascade power station, it is very
439 likely that the current population was introduced from Yangtze River.

440 However, the genetic diversity of the native Pearl River clades in both bighead
441 and silver carp (i.e. Clade II (Table 2) or Lineage II (Table 3)) was extremely low.
442 Moreover, the genetic diversity of native bighead and silver carp in the Pearl River
443 was lower compared to *Squaliobarbus curriculus*, an important economic species
444 mainly distributed in the Pearl River, which was surveyed at the same time and
445 showed high haplotype diversity and nucleotide diversity based on D-loop sequences
446 (h=0.982, π=0.01353) (Li *et al*., 2018). Other native economic freshwater fishes that
447 were not extensively cultured or introduced in the Pearl River, such as *Schizothorax*
448 *lissolabiatu* (Han *et al*., 2010) and *Culter alburnus* (Yang *et al*., 2016), also had a
449 higher genetic diversity revealed by the same molecular marker. Overall, our results
450 suggest that human activities such as introductions of Yangtze River populations, dam
451 constructions (river fragments), and stock enhancement activities have already had a
452 negative impact on the genetic diversity of native bighead and silver carp.

453

454 **4.3 | Implications for conservation and population management**

455 Knowledge on the amount of genetic diversity existing within and among
456 populations, its distribution through the species range and its temporal stability over
457 generations are key issues to design appropriate management and conservation
458 strategies (Vera *et al*., 2019). As two major domestic fishes in China and important

459 economic fishes in the Pearl River, bighead and silver carp have been used for
460 aquaculture and culture-based capture fisheries in natural systems for thousands of
461 years (Mao *et al*., 2010). However, due to the lack of proper resource assessments and
462 management plans, the released fry used for stock enhancement in Pearl River have
463 mainly been taken from hatchery stocks derived from the Yangtze River fish. It is
464 fortunate that we found a small number of native bighead and silver carp populations
465 in the Pearl River. However, Yangtze River populations have colonized all the
466 tributaries of the Pearl River system with the potential for hybridization with the
467 native Pearl River populations, which has also taken place in a similar manner in
468 Mediterranean brown trout (Splendiani *et al*., 2019). Moreover, a large number of the
469 fish fry of these two species derived from Yangtze River parents were released in the
470 Pearl River every year without assessment of their genetic background.

471 Even though the genetic diversity of these two carps in the Pearl River was
472 higher than in Yangtze River, this is likely due to the pronounced mitochondrial gene
473 flow from Yangtze River. Thus, in the light of this new knowledge, we propose that
474 stock enhancement should be stopped immediately until fish farms with native Pearl
475 River bighead and silver carp are founded. At the same time, regulatory rules and
476 actions on the fry sources used in releasing activities should be organized and
477 strengthened.

478 Meanwhile, we propose a wide-range survey focusing on the genetic resources of
479 native Pearl River bighead and silver carp based on both mtDNA and nuclear markers
480 (e.g., microsatellites, SNPs, nuclear genes), as has been performed in Yangtze River

and Western Europe. This kind of survey is fundamental to the establishment of national aquatic germplasm reserves for economically important species (Li *et al*., 1998; Geng *et al*., 2006; Wang *et al*., 2008; Berrebi *et al.*, 2019; Splendiani *et al*., 2019; Vera *et al*., 2019) and will determine if admixture is occurring or if the Yangtze-origin haplotypes are from released individuals. In the future, fish from the Pearl River should be used to preserve the genetic diversity of native carp populations. Moreover, based on the haplotype network analysis, population XJ had haplotypes clustered in Clade I and II (bighead carp) (Supporting Information Figure S1) and Lineages I and II (silver carp) (Supporting Information Figure S2). Therefore, Xijiang River could be an appropriate site for setting up a state-level reserve for native populations of various species in Pearl River, including two other carp species, black carp (*Mylopharyngodon piceus*) and grass carp (*Ctenopharyngodon idellus*), to provide high-quality germplasm not only for southern China but for the entire country and even the world.

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Author contributions

C. L., J. Z. and K. R. E. contributed to the study design; C. L. and J. Q. C. contributed to the sample preparation and molecular experiments; C. L. and K. S. performed the bioinformatic analyses; C. L., J. J. W., R. K. V. and K. S. wrote the paper with significant input from K.R.E.. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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692 **Tables**

693 **TABLE 1** Sampling locations, location codes, and sample sizes of bighead

694 *Hypophthalmichthys nobilis* and silver carp *Hypophthalmichthys molitrix.* XJYY: Fry

695 of silver carp sampled from the Xijiang River and raised for four months in laboratory.

696 Yangtze River*: Sequences downloaded from NCBI database.

697 **TABLE 2** Genetic diversity of bighead carp *Hypophthalmichthys nobilis* based on

698 D-loop sequences. Refer to Table 1 for the abbreviations of populations. Clade I and

699 Clade II are classified by the haplotype network analysis.

700 **TABLE 3** Genetic diversity of silver carp *Hypophthalmichthys molitrix* based on

701 D-loop sequences. Refer to Table 1 for the abbreviations of populations. Lineage I

702 and Lineage II are classified by the haplotype network analysis.

703 **TABLE 4** Matrix of genetic distance of bighead carp *Hypophthalmichthys nobilis*

704 based on D-loop sequences. Two sequences of silver carp sampled from the Pearl

705 River (HM01, HM02) were used as an out-group.

706 **TABLE 5** Matrix of genetic distance of silver carp *Hypophthalmichthys molitrix*

707 based on D-loop sequences. Two sequences of bighead carp sampled from the Pearl

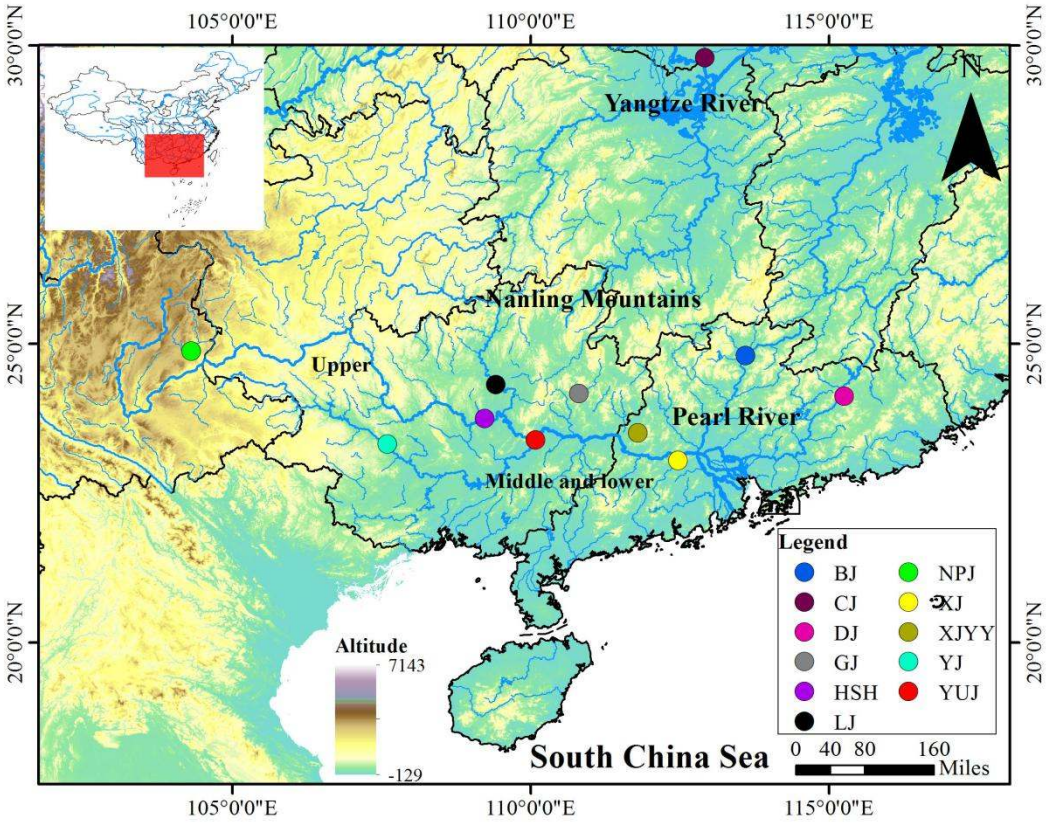
708 River (HN01, HN02) were used as an out-group.

709

710

Figures

FIGURE 1 Map showing the sampling sites of bighead carp *Hypophthalmichthys nobilis* and silver carp *Hypophthalmichthys molitrix* in the Pearl River. Refer to Table 1 for the abbreviations of populations. The map at the top left corner shows the relative location of the research area in China.

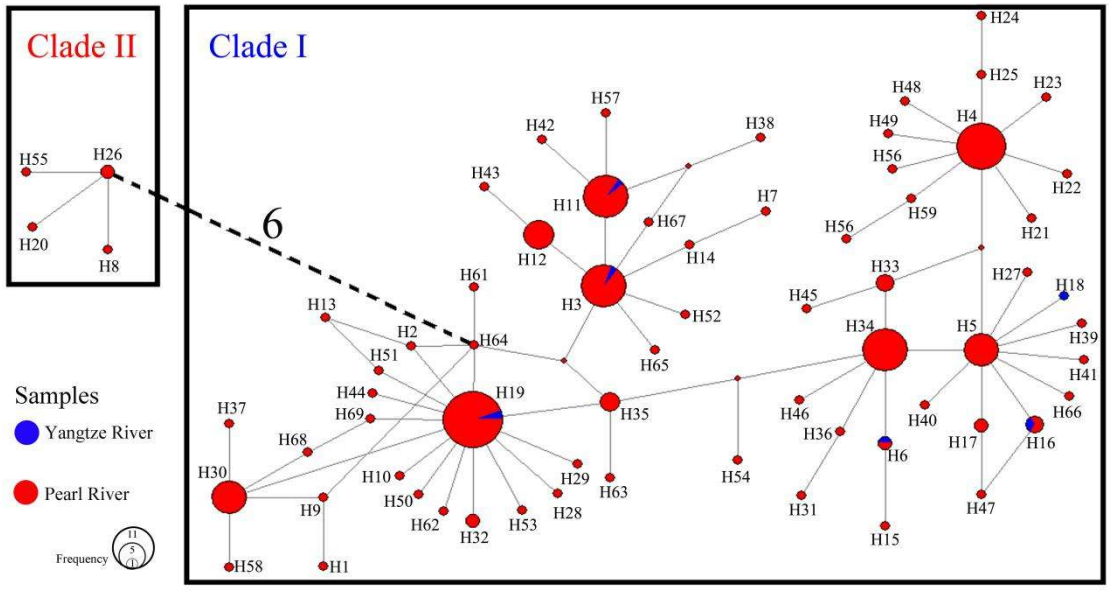


718 **FIGURE 2** The haplotype network of bighead carp *Hypophthalmichthys nobilis*

719 based on D-loop sequences. The arabic numerals near the dash line mean number of

720 mutations. Clade II is the native Pearl River genetic signature. Samples from the

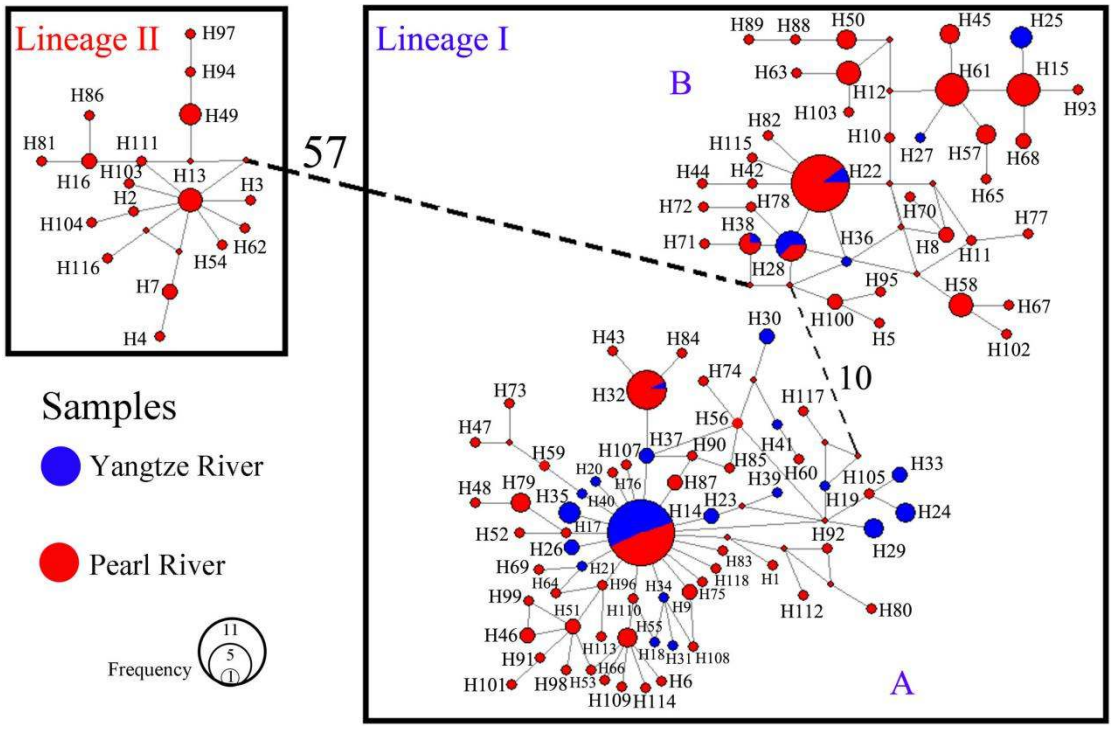
721 Yangtze River are coloured blue, samples from the Pearl River are coloured red.



722

723

724 **FIGURE 3** The haplotype network of silver carp *Hypophthalmichthys molitrix* based
725 on D-loop sequences. The arabic numerals near the dash line mean number of
726 mutations. Lineage II is the native Pearl River genetic signature. A, B are two
727 sublineages of lineage I. Samples from the Yangtze River are coloured blue, samples
728 from the Pearl River are coloured red.



729

Supporting Information

TABLE S1 Matrix of genetic distance (below diagonal) and genetic differentiation index *Fst* (above diagonal) of bighead *Hypophthalmichthys nobilis* based on D-loop sequences. BJ: Beijiang River; CJ: Yangtze River; DJ: Dongjiang River; GJ: Guijiang River; HSH: Hongshuihe River; LJ: Liujiang River; NPJ: Nanpanjiang River; XJ: Xijiang River; YJ: Youjiang River; YUJ: Yujiang River.

TABLE S2 Matrix of genetic distance (below diagonal) and genetic differentiation index *Fst* (above diagonal) of silver carp *Hypophthalmichthys molitrix* populations based on D-loop sequences. BJ: Beijiang River; CJ: Yangtze River; DJ: Dongjiang River; GJ: Guijiang River; HSH: Hongshuihe River; LJ: Liujiang River; NPJ: Nanpanjiang River; XJ: Xijiang River; XJYY: fry sampled from Xijiang River; YJ: Youjiang River; YUJ: Yujiang River.

TABLE S3 Demographic indices of bighead carp *Hypophthalmichthys nobilis* and silver carp *Hypophthalmichthys molitrix* based on D-loop sequences.

FIGURE S1 The haplotype network of bighead carp *Hypophthalmichthys nobilis* based on D-loop sequences. The arabic numerals near the dash line mean number of mutations. BJ: Beijiang River; CJ: Yangtze River, its haplotypes were indicated by the arrows; DJ: Dongjiang River; GJ: Guijiang River; HSH: Hongshuihe River; LJ: Liujiang River; NPJ: Nanpanjiang River; XJ: Xijiang River; YJ: Youjiang River; YUJ: Yujiang River.

FIGURE S2 The haplotype network of silver carp *Hypophthalmichthys molitrix* based on D-loop sequences. The arabic numerals near the dash line mean number of

752 mutations. BJ: Beijiang River; CJ: Yangtze River; DJ: Dongjiang River; GJ: Guijiang
753 River; HSH: Hongshuihe River; LJ: Liujiang River; NPJ: Nanpanjiang River; XJ:
754 Xijiang River; XJYY: fry sampled from Xijiang River; YJ: Youjiang River; YUJ:
755 Yujiang River.

756 **FIGURE S3** Mismatch distributions of bighead carp *Hypophthalmichthys nobilis* and
757 silver carp *Hypophthalmichthys molitrix* based on D-loop sequences. Light green
758 curves show the expected distribution of mutations according to the null hypothesis of
759 constant population size. The number of pairwise differences and their frequencies is
760 shown on the horizontal and vertical axes, respectively.

761

762 **Supporting Information**

763 **TABLE S1** Matrix of genetic distance (below diagonal) and genetic differentiation index *Fst* (above diagonal) of bighead carp

764 *Hypophthalmichthys nobilis* populations based on D-loop sequences. BJ: Beijiang River; CJ: Yangtze River; DJ: Dongjiang River; GJ: Guijiang

765 River; HSH: Hongshuihe River; LJ: Liujiang River; NPJ: Nanpanjiang River; XJ: Xijiang River; YJ: Youjiang River; YUJ: Yujiang River.

Population	BJ	CJ	DJ	GJ	HSH	LJ	NPJ	XJ	YJ	YUJ
BJ	-	-0.045	0.044	-0.008	-0.001	-0.024	0.037	0.013	-0.004	0.087
CJ	0.005	-	0.032	-0.061	-0.066	-0.046	-0.025	-0.031	-0.065	0.083
DJ	0.007	0.006	-	0.049	0.039	0.030	0.001	0.003	0.064	0.182
GJ	0.006	0.005	0.006	-	-0.038	-0.030	-0.003	-0.025	-0.043	0.109
HSH	0.005	0.004	0.006	0.004	-	-0.017	-0.008	-0.017	-0.019	0.124
LJ	0.006	0.006	0.007	0.006	0.005	-	0.003	-0.016	-0.013	0.060
NPJ	0.006	0.005	0.006	0.005	0.005	0.006	-	-0.016	0.015	0.165
XJ	0.006	0.006	0.007	0.006	0.005	0.007	0.006	-	-0.010	0.118

YJ	0.005	0.004	0.006	0.005	0.004	0.005	0.005	0.006	-	0.150
YUJ	0.005	0.004	0.006	0.004	0.004	0.005	0.005	0.005	0.004	-

766

767

768

769 **TABLE S2** Matrix of genetic distance (below diagonal) and genetic differentiation index *Fst* (above diagonal) of silver carp

770 *Hypophthalmichthys molitrix* populations based on D-loop sequences. BJ: Beijiang River; CJ: Yangtze River; DJ: Dongjiang River; GJ: Guijiang

771 River; HSH: Hongshuihe River; LJ: Liujiang River; NPJ: Nanpanjiang River; XJ: Xijiang River; XJYY: fry sampled from Xijiang River; YJ:

772 Youjiang River; YUJ: Yujiang River.

Population	BJ	CJ	DJ	GJ	HSH	LJ	NPJ	XJ	XJYY	YJ	YUJ
BJ	-	0.248	0.277	0.231	0.029	0.195	0.066	0.012	-0.037	0.192	0.099
CJ	0.034	-	0.402	-0.061	0.105	0.194	0.091	0.106	0.219	0.063	0.019
DJ	0.033	0.013	-	0.385	0.191	0.150	0.150	0.186	0.239	0.225	0.223
GJ	0.035	0.009	0.014	-	0.068	0.132	0.062	0.069	0.193	-0.027	-0.019
HSH	0.037	0.022	0.022	0.022	-	0.060	-0.020	-0.035	-0.006	0.038	-0.007
LJ	0.034	0.014	0.012	0.014	0.023	-	0.031	0.073	0.135	-0.007	0.076
NPJ	0.036	0.019	0.019	0.020	0.027	0.020	-	-0.016	0.027	0.011	-0.007
XJ	0.038	0.024	0.024	0.024	0.030	0.025	0.029	-	-0.010	0.054	-0.007

XJYY	0.038	0.030	0.029	0.030	0.033	0.029	0.033	0.035	-	0.134	0.070
YJ	0.034	0.012	0.013	0.012	0.022	0.013	0.020	0.025	0.029	-	0.011
YUJ	0.036	0.016	0.018	0.017	0.026	0.019	0.024	0.028	0.032	0.018	-

773 **TABLE S3** Demographic indices of bighead carp *Hypophthalmichthys nobilis* and
774 silver carp *Hypophthalmichthys molitrix* based on D-loop sequences.

Species	Group	Tajima's *D*	Fu's *Fs*	SSD	Harpending's Raggedness index
Bighead carp					
	Clade I	-2.06816*	-24.36304*	0.00778	0.01815*
	Clade II	-0.66823	-0.33158	0.03639	0.11000
Silver carp					
	Lineage I	-0.77187	-5.70386*	0.51856	0.00513
	Lineage II	-1.58286*	-23.94610*	0.01309*	0.02973

775 SSD: the sum of squared deviation. *Statistically significant results ($P < 0.05$).

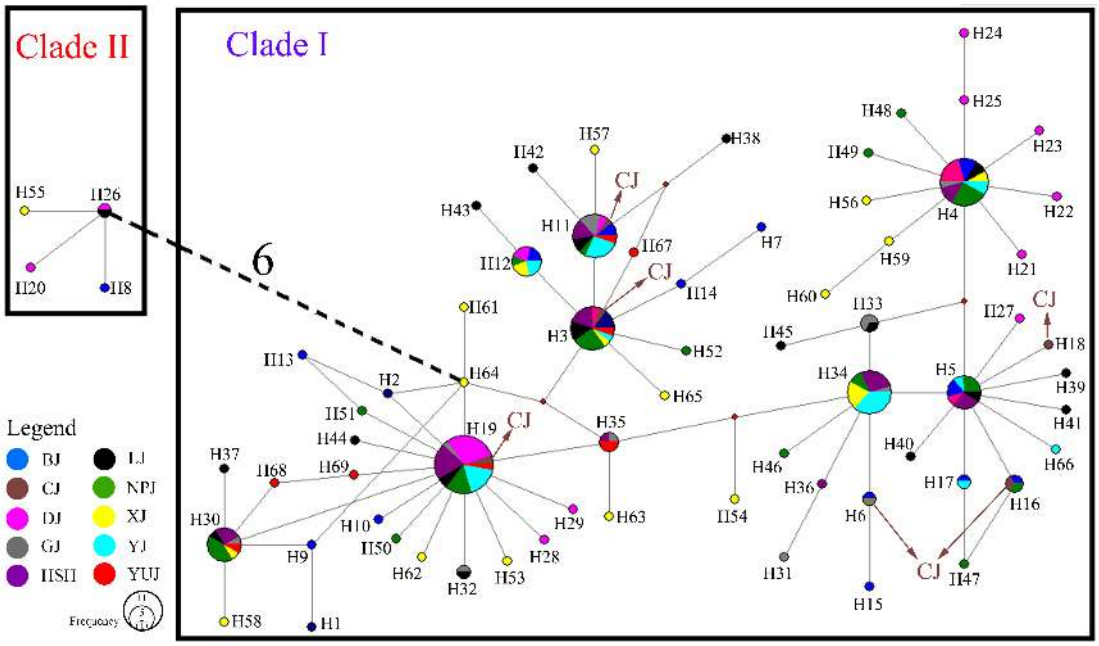


FIGURE S1 The haplotype network of bighead carp *Hypophthalmichthys nobilis* based on D-loop sequences. The arabic numerals near the dash line mean number of mutations. BJ: Beijiang River; CJ: Yangtze River, its haplotypes were indicated by the arrows; DJ: Dongjiang River; GJ: Guijiang River; HSH: Hongshuihe River; LJ: Liujiang River; NPJ: Nanpanjiang River; XJ: Xijiang River; YJ: Youjiang River; YUJ: Yujiang River.

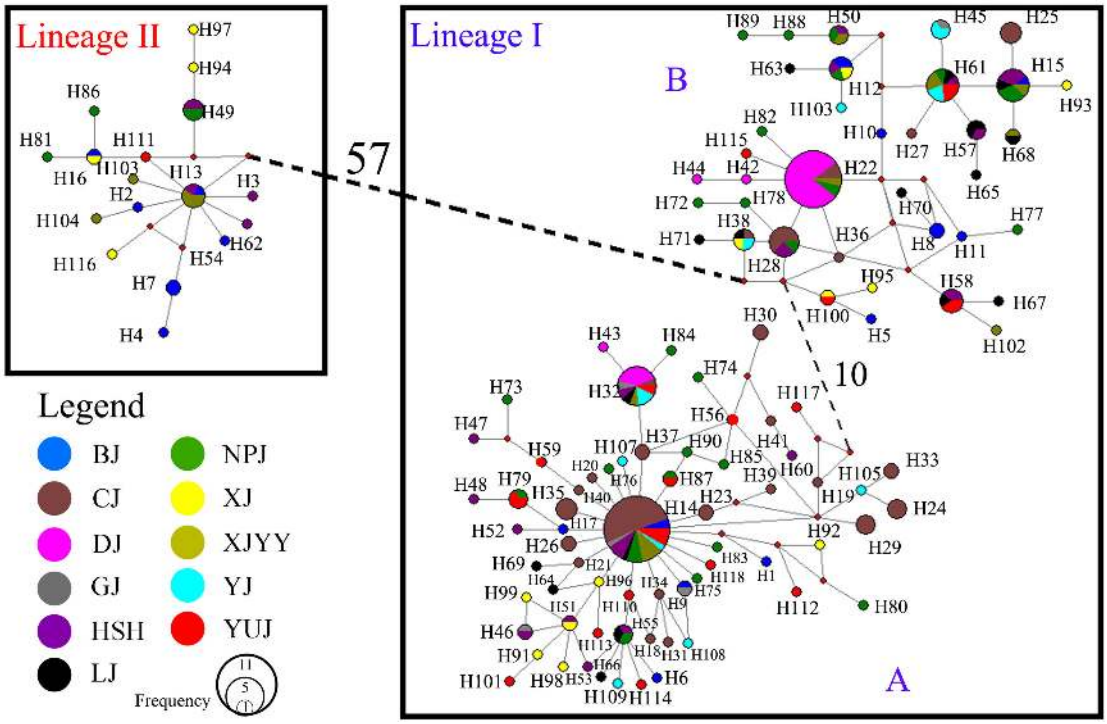


FIGURE S2 The haplotype network of silver carp *Hypophthalmichthys molitrix* based on D-loop sequences. The arabic numerals near the dash line mean number of mutations. BJ: Beijiang River; CJ: Yangtze River; DJ: Dongjiang River; GJ: Guijiang River; HSH: Hongshuihe River; LJ: Liujiang River; NPJ: Nanpanjiang River; XJ: Xijiang River; XJYY: fry sampled from Xijiang River; YJ: Youjiang River; YUJ: Yujiang River.