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Mitochondrial diversity and population structure of grass carp (*Ctenopharyngodon idella*) in the Pearl River after anthropogenic release

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Abstract – The grass carp *Ctenopharyngodon idella* is a commercially relevant carp species with a long-term artificial release history in China. To date, several genetic diversity studies have been performed on the Yangtze River *C. idella* populations, but similar reports were sparse for the Pearl River populations. Here, our study explored the genetic diversity patterns and population structure of the Pearl River *C. idella* populations after human intervention using two mitochondrial loci. Phylogenetic analyses demonstrated that grass carp populations in the Pearl River exhibited two maternal genetic lineages. Haplotype networks demonstrated that most main Pearl River haplotypes were shared with those of the Yangtze River samples. No genetic structure was detected among the Pearl populations and low level of population differentiation was observed between individual Yangtze River populations and the Pearl River populations. These findings might be attributed to the high dispersal ability of grass carp, as well as anthropogenic release. Moreover, the nucleotide diversity of the Pearl River populations was higher than that of the Yangtze River, indicating that artificial release programs might have significant effects on the genetic diversity of the Pearl River populations. Taken together, our findings demonstrated artificial release programs may have influenced the Pearl River grass carp populations and provide important knowledge that may guide the future management of grass carp in the Pearl River.

Keywords: Pearl River / grass carp / artificial release programs / genetic diversity / mitochondrial loci

1 Introduction

The grass carp *Ctenopharyngodon idella* (Valenciennes, 1844), a major commercial and native species in China, has a natural range that extends from the Pearl River to the Amur River (Li and Fang, 1990). This species has relatively strong dispersal ability and migrate upwards to upper and middle river section for reproduction (Chen, 1998). Grass carp has become a widely consumed freshwater fish species in China and has also been introduced to many countries for aquaculture, plankton control, and fisheries enhancement (Xie *et al.*, 2018). However, overfishing, dam construction, and environmental disruption have seriously reduced native grass carp populations and therefore several enhancement and release programs have been implemented to increase local stocks in many river sections (Li *et al.*, 2009b; Zhu *et al.*, 2009; Yang *et al.*, 2013). Four national breeding farms have been established and serve as the main supplier of carp fry, including *C. idella* (Zhang *et al.*, 2012). These farms select their broodstock from the Yangtze River, as these populations possess several desirable traits for aquaculture including a larger body size at maturity and a faster growth rate (Li, 1990).

The Pearl River is the second largest river in China and flows from west to east in the Southern China. This river has the highest fish diversity among Chinese rivers and has therefore been defined as a global hotspot of fish diversity (Xing *et al.*, 2016). A large number of dams built in the Pearl River significantly influences fish in this drainage (Fig. 1). For example, a famous large dam, *i.e.* Changzhou dam in Wuzhou, Guangxi province, China (build in 2007) has been deemed to have significant effect on fish community structure in the Pearl River (Li *et al.*, 2010; Shuai *et al.*, 2017). Many reports argued that native grass carp populations in this river have decreased rapidly due to overfishing and dam construction (Li *et al.*, 2009a, 2010; Shuai *et al.*, 2017). To enhance grass

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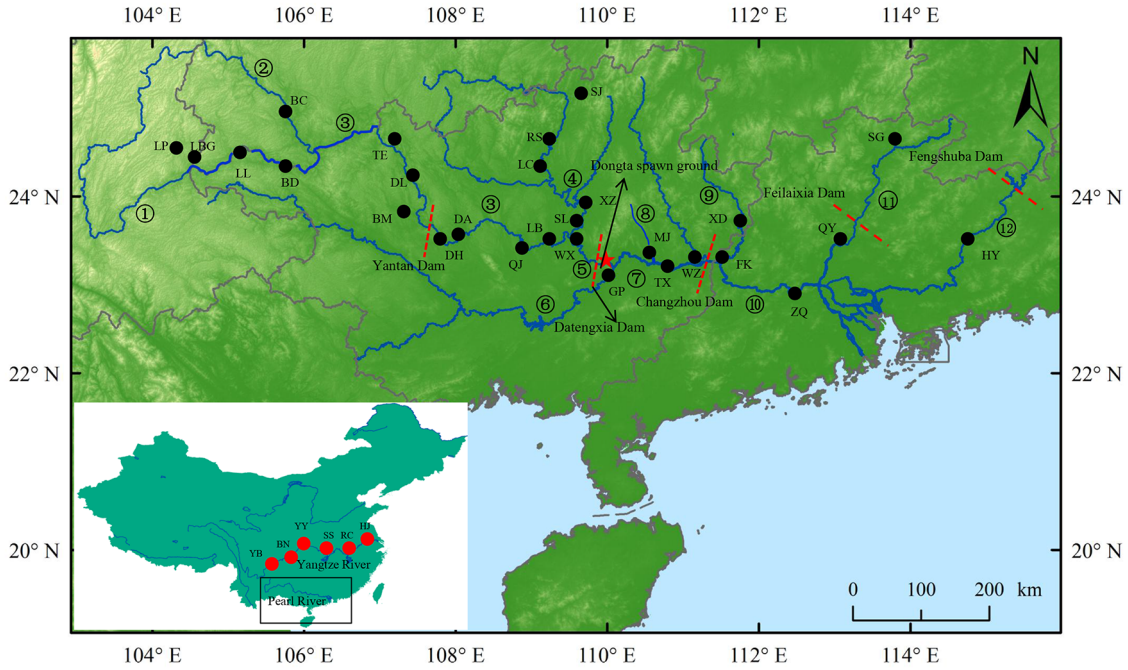

Fig. 1. Map of sampling locations of *Ctenopharyngodon idella*. The abbreviations correspond to the sampling locations and are detailed in Table S1. The circled numbers indicate 12 river sections within the Pearl River (1, Beipanjiang; 2, Nanpanjiang; 3, Hongshuihe; 4, Lijiang; 5, Qianjiang; 6, Yujiang; 7, Xunjiang; 8, Mengjiang; 9, Hejiang; 10, Xijiang; 11, Beijiang; 12, Dongjiang). The red star and dashed line represent Dongta spawning ground and Changzhou dam, respectively.

carp populations in the Pearl River, many local governments have conducted grass carp artificial enhancement and release programs since the beginning of 2000s in which carp fry obtained from Yangtze River breeders were released (Mao *et al.*, 2010). A previous report argued that the Yangtze River populations and the Pearl River populations showed different genetic clusters due to geographical isolation (Shen *et al.*, 2019). Artificial release programs may translocate non-native grass carps to non-native habitats, which might lead to genetic admixture (Splendiani *et al.*, 2016; 2019). To date, we knew little about the influences of artificial introduction on the native Pearl River populations.

Assessing the genetic diversity and population differentiation processes of particular taxa can provide crucial insights for the development of conservation strategies (Crandall *et al.*, 1999; Chen *et al.*, 2020). Several studies have previously assessed genetic variation and population differentiation levels in grass carp (Li, 1990; Zhang *et al.*, 2001; Liao *et al.*, 2005; Liu *et al.*, 2009; Zhao *et al.*, 2011; Shen *et al.*, 2019; Zhai *et al.*, 2020). However, most genetic studies mainly focused on grass carp populations in the Yangtze River or a few small populations in the Pearl River (Li, 1990; Zhang *et al.*, 2001; Liao *et al.*, 2005; Liu *et al.*, 2009; Zhao *et al.*, 2011; Shen *et al.*, 2019; Zhai *et al.*, 2020). Little is known about the genetic diversity and differentiation of the Pearl River populations. Past studies considered that native grass carp populations in the Pearl River arrived from the Yangtze and/or Qiantangjiang rivers during glacial periods (Li and Fang, 1990) and showed remarkable genetic differentiation between the Pearl River populations and the Yangtze River populations (Shen *et al.*, 2019). Given that artificial release programs have introduced novel Yangtze River populations into the Pearl River, we assumed that the genetic diversity and differentiation of the native grass carp populations of the Pearl River might be significantly impacted. Understanding the genetic diversity of this species in the Pearl River is necessary to test the effects of artificial introduction on native populations and can facilitate the design of future management strategies and policies.

The purposes of this study were (i) to analyze the genetic diversity and population structure of grass carp populations in the Pearl River, (ii) to test the effects of artificial release programs on the native Pearl River populations, and (iii) to offer meaningful implications for conservation on the Pearl River populations.

2 Materials and methods

2.1 Sampling

Grass carp specimens were obtained from local fishermen in the Pearl River between 2010 and 2019 (Tab. S1). A small piece of fin or muscle tissue was clipped from each specimen and stored in 99% ethanol. The total genomic DNA was extracted from fin or muscle tissues using the Axygen DNA Extraction Kit and examined via electrophoresis in 1% agarose gel. The partial mitochondrial cytochrome *b* gene (Cytb) and control region (Dloop) were amplified for all specimens using the L14724 and H15915 (Xiao *et al.*, 2001) and DL1 and DH2 universal primers (Liu and Chen, 2003), respectively. The amplification of the two gene fragments was conducted using the PCR conditions described by Chen *et al.* (2020). The amplified fragments were purified using 1.2% low-melting agarose gel electrophoresis and sequenced with the above-

described primer pairs using an ABI PRISM 3700 (Applied Biosystems) automatic DNA sequencer. All sequences were deposited in GenBank under accession numbers MZ815441-MZ816036.

Cytb and Dloop fragments of 58 grass carp individuals from the Yangtze River analyzed by Zhao *et al.* (2011) were also used (Tab. S1). Both the Pearl River and Yangtze River samples were stored for downstream analysis.

2.2 Sequence analyses

Novel nucleotide sequences were initially edited using SEQMAN in DNASTAR (DNASTAR Inc., Madison, WI, USA). All sequences were aligned using MUSCLE (Edgar, 2004) and then visually examined using MEGA version 6.0 (Tamura *et al.*, 2013). Cytb and Dloop were concatenated as one mitochondrial locus (MCD) in our analyses. Identical haplotypes for MCD were collapsed using DNASP version 5.0 (Librado and Rozas, 2009). A haplotype network was built using the HAPLOVIEW software (Barrett *et al.*, 2004).

2.3 Phylogenetic analyses and estimation of divergence time

Phylogenetic analyses were conducted using BEAST version 1.8.1 (Drummond and Rambaut, 2007) and *Mylopharyngodon piceus* was selected as the outgroup. The analyses were executed for one hundred million generations under a strict clock model based on Yule processes and Random starting topologies. The optimal substitution model (HKY + I + G) was chosen based on the Akaike information criterion (AIC) using MrModeltest version 2.3 (Nylander, 2004). A substitution rate range of 1.0–2.0% per million years is often adopted for the Cytb gene in cyprinid fish (Meyer, 1993; Ketmaier *et al.*, 2004). Therefore, we estimated substitution rates of 1.26% and 2.52% substitutions/million years for MCD using the ratio of Kimura's (1980) two-parameter (K2P) distance (Kimura, 1980) between MCD and Cytb. The average net K2P distance between the MCD and Cytb alone was 1.26. Therefore, the estimated mean substitution rate for MCD was calculated by multiplying the Cytb rate of cyprinid fish (1.0–2.0%) by the ratio for Cytb alone (1.26). The phylogenetic trees were sampled every 1000 generations, resulting in 100000 trees, and the first 25% were discarded as burn-ins. The effective sample sizes (ESSs) (>200) for the parameter estimates and convergence were assessed in Tracer 1.5 (Drummond *et al.*, 2012). The final trees were summarized in TreeAnnotator 1.8.1 (Drummond *et al.*, 2012).

2.4 Molecular diversity and population structure

The number of haplotypes (n), haplotype diversity (h), and nucleotide diversity (π) were calculated in DNASP version 5.0 (Librado and Rozas, 2009). A regression analysis was performed for each river section in the Pearl River and for each population in the Yangtze River to assess the relationship between sample size and genetic diversity indexes including haplotype diversity and nucleotide diversity. Furthermore, spatial distribution of haplotype diversity and nucleotide diversity was summarized using the Spatial Analyst extension in ARCMAP version 10.3 (ESRI, Redlands, CA, USA) and the ordinary Kriging method. The genetic distance between different lineages was estimated using MEGA version 6.0 based on the K2P model. Pairwise population differentiation (fixation index; ϕ_{ST}) was calculated in ARLEQUIN version 3.5 (Excoffier and Lischer, 2010). Analysis of molecular variance (AMOVA) was conducted in ARLEQUIN version 3.5 to infer the genetic structure of the Pearl River populations. All calculations implemented in ARLEQUIN version 3.5 were performed with a Jukes-Cantor distance and 1000 permutations.

3 Results

3.1 Sequence analysis

A total of 298 grass carp specimens from 28 locations covering main river sections within the Pearl River were collected and two mitochondrial fragments were successfully genotyped. After combining with the 58 Yangtze River samples, the Cytb fragment (1111 bp) contained 13 variable sites and the Dloop fragment (766 bp) had 17 variable sites. A total of 356 MCD sequences with a 1877 bp length and 41 haplotypes were identified (Tab. 1).

3.2 Phylogenetic analyses and haplotype networks

Phylogenetic analysis revealed two distinct lineages (A and B) for grass carp in both the Pearl River and the Yangtze River (Fig. 2). The K2P distance between lineage A and lineage B was 0.0046. BEAST analyses indicated the divergence times between A and B (0.135–0.270 Ma; Fig. 2). The haplotype network also revealed two haplotype groups (A and B), which was consistent with the phylogenetic results (Fig. 3; Fig. S1). The majority of the main haplotypes were shared by multiple populations. Furthermore, we found that 14 out of 18 Yangtze River haplotypes (highlighted in red) were mixed with the Pearl River haplotypes. Most Yangtze River samples belonged to group A (corresponding to lineage A) and five Yangtze River samples were found to cluster with group B (corresponding to lineage B) (Fig. 3).

3.3 Population genetic structure

Hierarchical AMOVA showed a moderate global ϕ_{CT} between the Pearl River populations and the Yangtze River populations (ϕ_{CT} = 0.15, $P < 0.001$; Tab. 2), whereas none genetic differentiation was detected among populations within each drainage (Tab. 2). Nonhierarchical AMOVA demonstrated an absence of genetic structure among the Pearl River populations (ϕ_{CT} = 0.00, P = 0.758; Tab. 2) and among the Yangtze River populations (ϕ_{CT} = 0.09, P = 0.030; Tab. 2). Significant differentiation was detected in 38 out of 66 pairwise ϕ_{ST} comparisons between the Yangtze River populations and the Pearl River populations with ϕ_{ST} values ranging from 0.099 to 0.570 (Tab. S2). Pairwise ϕ_{ST} comparisons among the Pearl River populations indicated that no ϕ_{ST} were significant (Tab. S2).

Table 1. Genetic diversity statistics for both the Pearl River and the Yangtze River populations. N, individual numbers; n, haplotype numbers; *h*, haplotype diversity; π, nucleotide diversity.

River section	N/n	*h*	π (%)
Nanpanjiang (1)	19/5	0.766 ± 0.059	0.215 ± 0.0003
Beipanjiang (2)	2/2	NA	NA
Hongshuihe (3)	21/13	0.919 ± 0.042	0.251 ± 0.0002
Liujiang (4)	35/11	0.844 ± 0.038	0.240 ± 0.0002
Qianjiang (5)	6/3	0.600 ± 0.215	0.263 ± 0.0009
Yujiang (6)	27/10	0.852 ± 0.048	0.262 ± 0.0002
Xunjiang (7)	42/11	0.836 ± 0.036	0.260 ± 0.0002
Mengjiang (8)	22/8	0.766 ± 0.079	0.246 ± 0.0002
Hejiang (9)	18/7	0.804 ± 0.069	0.257 ± 0.0003
Xijiang (10)	50/12	0.830 ± 0.029	0.241 ± 0.0001
Beijiang (11)	38/9	0.822 ± 0.033	0.244 ± 0.0001
Dongjiang (12)	18/8	0.699 ± 0.117	0.228 ± 0.0004
Pearl River	298/41	0.821 ± 0.014	0.245 ± 0.0001
Yibin (YB)	10/2	0.467 ± 0.132	0.025 ± 0.0001
Banan (BN)	10/5	0.756 ± 0.130	0.232 ± 0.0007
Yunyang (YY)	9/2	0.500 ± 0.128	0.027 ± 0.0001
Shishou (SS)	10/4	0.778 ± 0.091	0.205 ± 0.0007
Ruichang (RC)	9/3	0.639 ± 0.126	0.042 ± 0.0001
Hanjiang (HJ)	10/4	0.733 ± 0.120	0.110 ± 0.0005
Yangtze River	58/18	0.723 ± 0.041	0.118 ± 0.0003

3.4 Molecular genetic diversity

Our findings indicated that the Pearl River populations had a high overall haplotype diversity (H*d* = 0.821) and comparatively low global nucleotide diversity (π = 0.245%; Tab. 1). In contrast, the global haplotype diversity and nucleotide diversity of the Yangtze River populations were 0.723 and 0.118%, respectively. Haplotype diversity and nucleotide diversity varied among localities in the Pearl River, ranging from 0.600 to 0.919, and from 0.215% to 0.263%, respectively (Tab. 1). The haplotype diversity and nucleotide diversity of the Yangtze River populations ranged from 0.467 to 0.778, and from 0.025% to 0.232%, respectively (Tab. 1). A regression analysis revealed that the haplotype diversity was significantly correlated with sample size (Spearman correlation analysis: P = 0.002), whereas nucleotide diversity was not significantly correlated with sample size (Spearman correlation analysis: P = 0.142). We found that middle reaches showed the relatively higher haplotype and nucleotide diversities (Fig. 4).

4 Discussion

4.1 Genetic diversity and genetic structure

Two distinct haplotype lineages were observed in both the Yangtze River and the Pearl River based on phylogenetic analyses and a haplotype network, suggesting that grass carps experienced long-term geographic isolation. Previous studies have reported that grass carp in the Yangtze River had two mtDNA lineages (Lu *et al.*, 1997; Zhao *et al.*, 2011; Zhai *et al.*, 2020). This finding is consistent with those reported for bighead carp *Hypophthalmichthys nobilis* and silver carp *Hypophthalmichthys molitrix* in the Pearl River, both of which also present two mtDNA lineages in the Pearl River (Li *et al.*, 2020). However, a larger divergence was detected in bighead carp and silver carp than that in grass carp, suggesting that two *Hypophthalmichthys* species likely underwent a more complex evolutionary history. Based on divergence time estimates, the two lineages likely split 0.135–0.270 million years ago, suggesting that grass carp underwent late Pleistocene divergence. Our study is the first to characterize the maternal lineage composition of grass carp in the Pearl River.

In this case study, genetic diversity was higher in the middle reaches of the Pearl River, indicating that this region could be treated as a genetic diversity hotspot of grass carp in the Pearl River (Fig. 4). Two potential reasons could explain this result. First, this region presents several river sections, like the Hongshuihe, Liujiang, Qianjiang, Yujiang and Xunjiang rivers (Fig. 1) and offers various suitable habitats for the grass carp populations, which can harbor larger population sizes and results in higher genetic diversity. Second, this region is close to Dongta spawning ground, which is the most important spawning ground for grass carp in the Pearl River (Shuai *et al.*, 2016). Therefore, this region can gather many breeding populations from different river sections and harbor higher genetic diversity.

According to AMOVA and ϕ_{ST} analyses, no genetic structure was detected among the Pearl River populations. The fact that the main haplotypes were shared by most of the populations also confirmed this observed pattern (Fig. 3). Anthropogenic release could potentially explain this phenomenon. A large number of juvenile grass carp have been released into many river sections within the Pearl River each year. Most of the released specimens had a genetic background which was consistent with that of the Yangtze River populations (Mao *et al.*, 2010). As more carp fry continue to be released into each river section, the genetic resources will likely become increasingly homogenized.

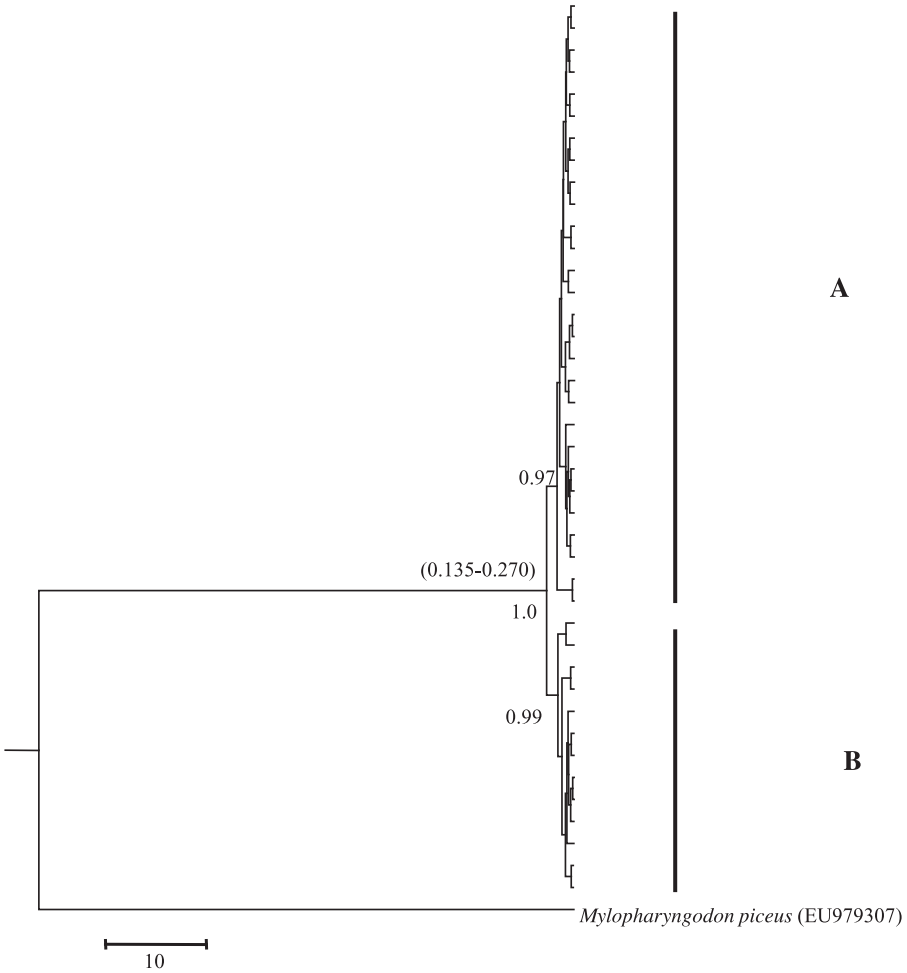

Fig. 2. Phylogenetic tree based on Bayesian inference showing the relationships among *Ctenopharyngodon idella* populations. The values on branches indicate the Bayesian posterior probabilities. The numbers in brackets indicate the estimated divergence time using different evolutionary rates.

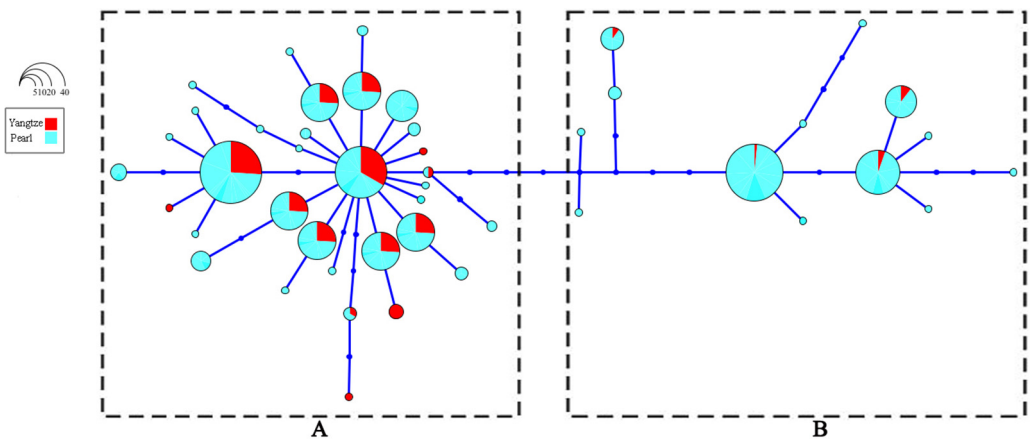

Fig. 3. Haplotype networks for the Pearl River population and the Yangtze River population based on the concatenated sequences of Cytb and Dloop (MCD). The circles are proportional to the sample size (the scale varies between networks) and are colored according to the different river populations.

Table 2. Analysis of molecular variance (AMOVA) results for different grouping options of *Ctenopharyngodon idella* populations estimated using ϕ–statistics based on the concatenated sequences of Cytb and Dloop (MCD).

Source of variation	Percentage of variation	ϕ_{CT}	*P*
Group by drainages			
Among drainages	15.33	0.15	<0.001
Among populations within drainage	–0.41	0	
Within populations	85.08	0.85	
Group by Pearl River populations			
Among populations	–1.01	0.00	0.758
Within populations	101.01	1.00	
Group by Yangtze River populations			
Among populations	8.53	0.09	0.03
Within populations	91.47	0.91	

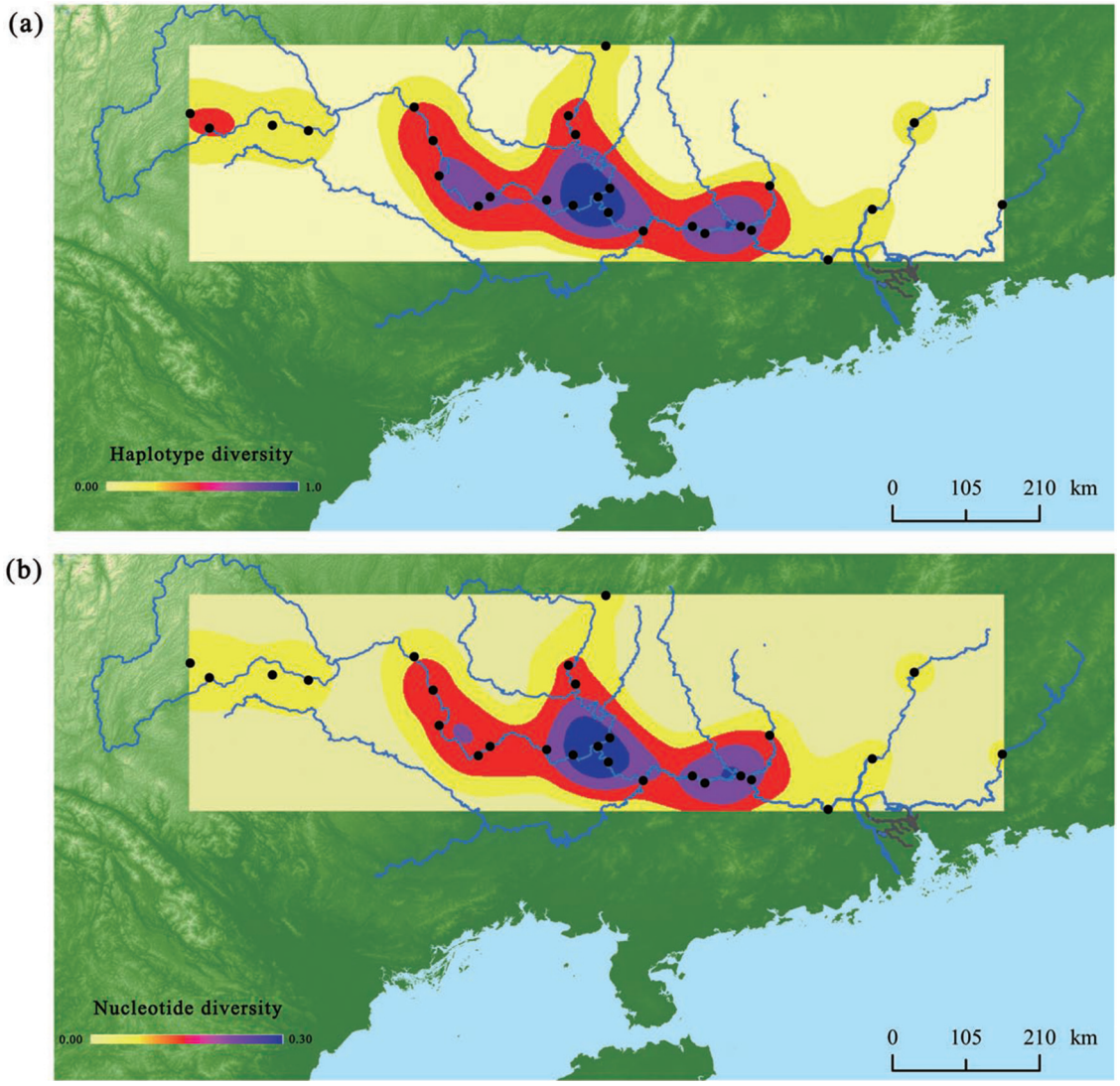

Fig. 4. Estimated distribution of haplotype and nucleotide diversity across the sample range of *Ctenopharyngodon idella*. (a) Haplotype diversity. (b) Nucleotide diversity. Blue indicates higher values of genetic diversity, while Yellow represents lower values.

4.2 Potential effects of anthropogenic introduction on the Pearl River populations

As shown in the haplotype network, most main haplotypes of the Pearl River were shared with those of the Yangtze River samples (Fig. 3), suggesting that detected haplotypes were likely introduced from the Yangtze River. The observed pattern was most likely caused by the colonization from the Yangtze River rather than shared ancestral polymorphisms, as explained below. Firstly, the samples from the hatcheries that produced the fry used for anthropogenic release activities mainly originated from the Yangtze River (Mao *et al.*, 2010). If populations from the two drainages shared ancestral polymorphisms, the two drainages will own a certain number of private haplotypes. However, main observed haplotypes were found to be shared by the two drainage individuals (Fig. 3). Secondly, our study found that low genetic differentiation was detected between some Yangtze River populations and many Pearl River populations, indicating that amounts of samples in the Pearl River come from the Yangtze River. Past reports highlighted that significant genetic differentiation has occurred among the native populations of the two drainages (Shen *et al.*, 2019). Thirdly, grass carp preferentially migrate to the upper river section for spawning and their eggs require a sufficient amount of accumulated temperature to hatch (Mao *et al.*, 2010), meaning that grass carp cannot complete their entire life history in fragmented rivers resulting from dam construction. Given that a large number of dams have been constructed in the Pearl River, grass carp populations in many river sections were mainly derived from human release. In fact, the populations that were separated by large spatial distances (e.g., the NP and DJ populations) shared most of their haplotypes with the Yangtze River samples (Fig. S1), providing a line of direct evidence of introduction.

This study only compared the nucleotide diversity between the Pearl River populations and the Yangtze River populations since haplotype diversity was correlated with sample size. Our findings indicated that the nucleotide diversity of the Pearl River was higher than that of the Yangtze River (Tab. 1), suggesting that anthropogenic introduction may enhance the nucleotide diversity of the Pearl River. If the grass carp populations of the two drainages migrated reciprocally without human intervention or the Pearl River populations originated from the Yangtze River during glacial periods, the two drainages would exhibit similar levels of nucleotide diversity or the nucleotide diversity of the Pearl River populations would be lower than that of the Yangtze River populations. Human introduction of grass carp from the Yangtze River brought novel genetic resources into the Pearl River and thus elevated the nucleotide diversity of the Pearl River. This pattern was also seen in bighead carp and silver carp (Li *et al.*, 2020). Furthermore, our study indicated that the nucleotide diversity of grass carp was much lower than that of other native carp species such as bighead carp and silver carp, which was also demonstrated in a previous study (Lu *et al.*, 1997).

4.3 Implications for future management

Although the genetic diversity of grass carp in the Pearl River was higher than that of the Yangtze River, this was likely due to the artificial introduction of Yangtze River genetic resources into the Pearl River. If large numbers of grass carp fry derived from Yangtze River broodstock continue to be released into the Pearl River without assessing their genetic background, native Pearl River populations will become homogeneous and private haplotypes in the Pearl River will disappear. Therefore, native Pearl River grass carp hatcheries should be established to restock the Pearl River and the genetic background of the fish fry should be evaluated before being released. Unlike previous mtDNA genetic analyses of bighead carp and silver carp in the Pearl River, native haplotype lineages of grass carp in the Pearl River will not be identified based on mtDNA loci. Instead, SNPs could be used to differentiate the native populations from the Yangtze River and the Pearl River (Shen *et al.*, 2019). This approach could be employed to identify native Pearl River grass carp, thus facilitating the development of fish farms with native grass carp. Furthermore, given that higher genetic diversity was observed in the middle reaches of the Pearl River, we suggested that more attention should be focused on grass carp populations distributed in this region.

Supplementary Material

The Supplementary Material is available at https://www.kmae.org/10.1051/kmae/2022012/olm.

Table S1 Summary of sample localities for *Ctenopharyngodon idella*. The locality, coordinates (longitude/latitude), voucher number, and GenBank accession number for Cytb and dloop are presented. The italicized Genbank numbers are the sequences from the Yangtze River obtained from a previous study by Zhao *et al.* (2011).

Table S2 Pairwise ϕ–statistics (ϕST) among the Yangtze River populations and the Pearl River populations. The values in bold indicate statistically significant ϕST.

Figure S1 Haplotype networks for each Pearl River population and the overall Yangtze River population based on concatenated sequences of Cytb and Dloop (MCD). The circles are proportional to the sample size (scale differs between networks) and are colored according to the different river section populations (See Fig. 1).

Conflict of interest

The authors declare no competing financial interest.

Data availability statement

DNA sequences have been deposited in GenBank under Accession numbers MZ815441-MZ816036. Details regarding individual samples are available in Table S1.

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