# Mitochondrial DNA diversity, population structure, and conservation genetics of four native carps within the Yangtze River, China

**Guoqing Lu, Sifa Li, and Louis Bernatchez**

**Abstract**: Silver carp (*Hypophthalmichthys molitrix*), grass carp (*Ctenopharyngodon piceus*), bighead carp (*Aristichthys nobilis*), and black carp (*Mylopharyngodon piceus*) rank first, second, fourth, and seventh in world fish production. In China, the Yangtze River harbours the most important natural populations of these species. We performed a polymerase chain reaction – restriction fragment length poymorphism analysis on 365 juvenile fish representing three nursery grounds to provide a first assessment of the mitochondrial DNA diversity in these species and test the hypothesis that they are composed of more than one genetic stock. The mitochondrial DNA diversity was high in silver, bighead, and black carp, and much less in grass carp. Analysis of heterogeneity of genotype frequency, fixation indices, intersite molecular variance, and localization indices indicated that juvenile silver, bighead, and black carp from different nursery areas belong to genetically distinct populations. These results suggest that their population structure may be determined by the number of environmental settings that permit closure of their life cycle. They also imply that carp from the Yangtze River cannot be managed as a single unit and that human disturbance through exploitation and habitat modifications, in particular the construction of the Three Gorges Dam, will have differential impacts on fish abundance for different parts of the river.

**Résumé** : *Hypophthalmichthys molitrix*, *Ctenopharyngodon piceus*, *Aristichthys nobilis*, et *Mylopharyngodon piceus* sont des poissons se rangeant premier, second, quatrième et septième au niveau de la production mondiale. En Chine, les populations les plus abondantes de ces espèces se retrouvent dans le fleuve Yangtze. Nous avons réalisé une première étude de la diversité de l'ADN mitochondrial afin de vérifier l'hypothèse voulant que ces espèces soient composées de plus d'un stock génétique. Une analyse PCR-RFLP d'un segment de l'ADN mitochondrial a été réalisée sur 365 spécimens provenant de trois aires d'alimentation juvénile. Une diversité élevée a été détectée chez toutes les espèces, sauf *C. piceus*. L'analyse de l'hétérogénéité des fréquences génotypiques, les estimés de l'indice de fixation, de la variance moléculaire entre sites et de l'indice de localisation ont révélé que les juvéniles provenant de différents aires d'alimentation appartiennent à des populations génétiquement différenciées. Ces résultats suggèrent que la structure populationnelle de ces espèces est en partie déterminée par le nombre d'entités environnementales à l'intérieur desquelles leur cycle vital peut être complété dans le fleuve Yangtze. Ces résultats impliquent que chaque espèce de carpe de ce fleuve ne peuvent être gérée comme un seul stock et que les perturbations anthropogéniques, notamment par l'exploitation et les modifications d'habitat telles que celles imposées par la construction du barrage des Trois Gorges, auront un effet différentiel sur leur abondance dans différentes sections du fleuve.

## Introduction

Silver carp *(Hypophthalmichthys molitrix)*, grass carp *(Ctenopharyngodon piceus)*, bighead carp *(Aristichthys nobilis)*, and black carp *(Mylopharyngodon piceus)* are cyprinids native to China (László et al.1992). Because of their growth performance and trophic specializations, these four species are highly suited for pisci-polyculture, and consequently have become the backbone of aquaculture in many Asian and eastern European countries (Freshwater Fish Culture Experimental Committee 1973; Lin 1982; László et al. 1992; Li and Mathias 1994). Thus, they rank first, second, fourth, and seventh, respectively, in world fish production (Li 1993). In China, they contribute together to more than 75% of the total freshwater fish production (Li 1993).

The Yangtze River harbours the most important natural populations of these species, where they have long been exploited by commercial fishing of older juvenile and adult fish and by massive fry collection to support fish farm production. It has also been shown that Chinese carps of the Yangtze River were generally superior to those from other Chinese rivers in terms of aquaculture performance (Li 1990; Li et al. 1990). Since the breakthrough of domestic reproduction in 1960s, wild fry are no longer the unique seed source for fish farming but still represent the major source of broodstock fish. Thus, fry of the four species are collected in nursery areas and raised to produce brood stocks. These are then used to produce one generation of fry that are sold to fish farms and raised for food. Many potential nursery areas have been identified along the river. However, Penyanghu and Tongtinghu lakes (the first and second largest in China, respectively), and nearby stretches of the Yangtze River into which they drain, are believed to represent the most important areas (Fig. 1).

Over the last two decades, a major reduction in recruitment,

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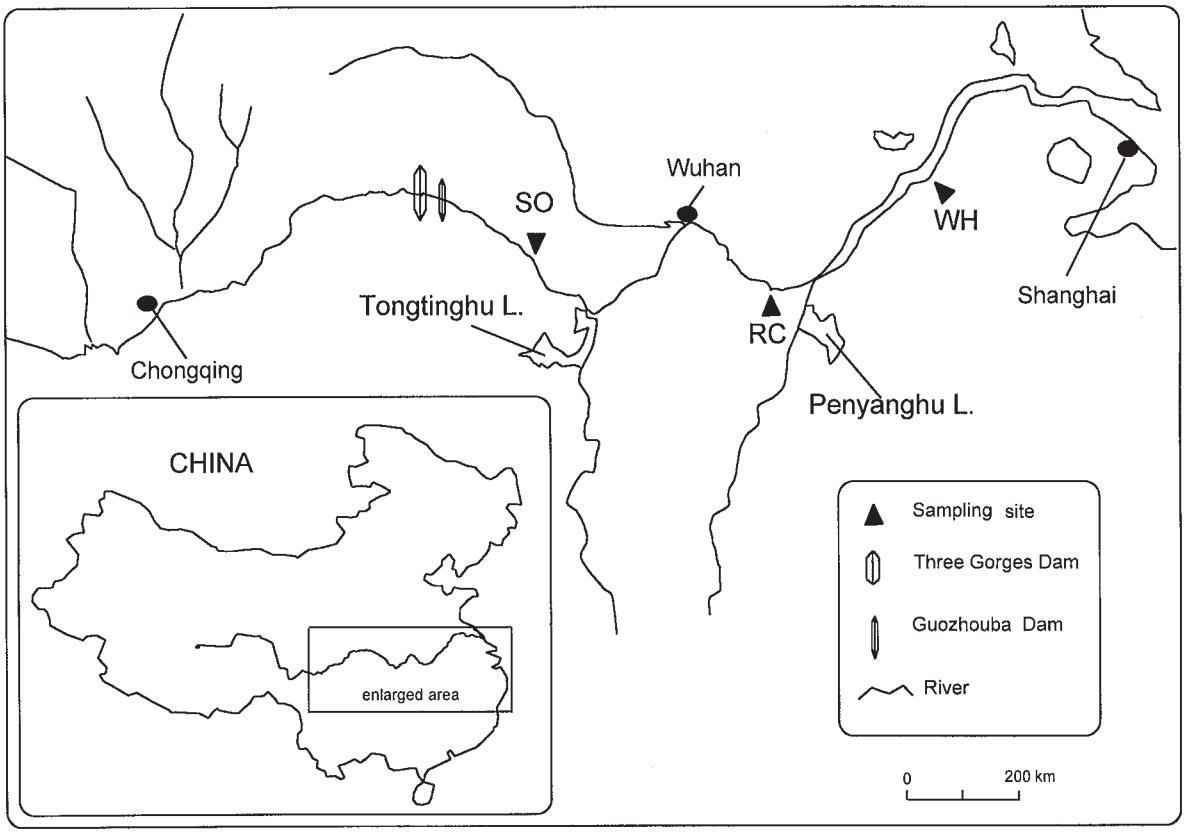
**Fig. 1.** Location map with sampling sites for four carp species from the Yangtze River. SO, Swan Oxbow; RC, Ruichang; WH, Wuhu.




most probably resulting from overfishing, water pollution, and dam construction, has been reported for all four species. Since the early 1980s, the abundance of wild fry has declined to less than 20% of that generally maintained historically (Survey Team 1982; Yi et al. 1988; Li et al. 1990). The ongoing construction of the world's biggest hydroelectric scheme, the Three Gorges Dam, will destroy major spawning grounds located downstream of the Guozhouba Dam (Fig. 1). These are believed to contribute to more than 40% of the whole river fry production (Yi et al. 1988). Consequently, there is little doubt that such a loss of reproductive habitat will cause further decline of wild carp populations.

To preserve the four species and compensate for the loss of natural fry production, the Chinese government founded a genetic resource pool by collecting fry of the four species from nursery areas downstream of the Yichang spawning grounds, and reared them in two nearby large oxbows to create brood stocks for captive breeding and fry production. The rationale for maintaining a genetic resource pool with fish from a single origin of the Yangtze River has recently been supported by electrophoretic analyses of genetic variation at enzymatic loci (Zhao and Li 1995). This study revealed no significant patterns of heterogeneity in allele frequency distribution among samples collected in several nursery areas distributed over 800 km, from the middle to the lower stretches of the river, suggesting that the four carp species are each composed of a single, homogeneous stock. However, an alternative hypothesis is that multiple stocks were undetected because of an insufficient level of resolution of the marker used (Hillis and Moritz 1990). Further investigations with a different genetic approach should allow a clearer investigation of the alternative hypothesis and provide additional knowledge that will help to orient management decisions related to the conservation genetics of these species (Carvalho and Hauser 1994).

Over the past decade, restriction fragment length polymorphism (RFLP) analysis of mitochondrial DNA (mtDNA) has been successfully used to assess population structure in fishes and has often revealed more pronounced genetic heterogeneity than isozyme analysis (reviewed in Billington and Hebert 1991). More recently, the automation of the polymerase chain reaction (PCR) (Saiki et al. 1985) and the design of universal or multispecific primers (e.g., Kocher et al. 1989; Meyer 1994) have permitted mtDNA analysis to be performed on selected segments of the mitochondrial genome from degraded or small amounts of tissues, further enhancing the usefulness of the method (e.g., Chow et al. 1993; Cronin et al. 1993; Chapman et al. 1994; Giuffra et al. 1994).

In this study, we performed a PCR-RFLP analysis of mtDNA diversity in silver, bighead, grass, and bighead carp to (*i*) assess of the usefulness of mtDNA diversity analysis in these species, and (*ii*) test the null hypothesis that each species is composed of a single gene pool in the Yangtze River. We then discuss the implications of the observed genetic population structure for the management and conservation genetics of these species in the face of exploitation and habitat disturbance within the Yangtze River.

## Materials and methods

A total of 365 juvenile fish (ranging from 160 to 320 mm in total

length and from 100 to 600 g) of silver, bighead, grass, and black carp were collected between September 1993 and February 1995 at three sampling sites located midstream (Swan Oxbow), mid-downstream (Ruichang section), and downstream (Wuhu section) on the Yangtze River (Fig. 1). These sites correspond to major nursery areas for the four species and are located downstream of the nearest spawning grounds. Fresh livers (1–3 g) were sampled and preserved in 95% ethanol until DNA purification.

Total DNA was purified following the method described in Bernatchez et al. (1992). Three mtDNA genes including the ND5 and ND6 subunits of the NADH dehydrogenase gene, cytochrome *b* gene, and the noncoding control region were amplified by the PCR in a Perkin-Elmer thermal cycler (model 480) using the primers C-Glu, its complementary sequence (which we named V-Glu), and C-leu3, designed by Park et al. (1993), as well as HN20 designed by Bernatchez and Danzmann (1993). C-Glu and C Leu3 amplified a segment encompassing the ND5/6 region (approximately 2400 base pairs (bp)) whereas V-Glu and HN20 amplified the cytochrome *b* gene and the control region (approximately 2100 bp). PCR conditions were as described in Bernatchez et al. (1995), except that the annealing temperature was lowered to 45°C for amplifying the cytochrome *b* – D-loop segment.

Restriction enzymes to be used for PCR-RFLP analysis were selected by first screening a total of 19 enzymes on five fish from each of the upstream and downstream locations for each species. The set of enzymes was the same as described by Bernatchez and Osinov (1995). Ten enzymes *(Alu*I, *Ava*II, *Dde*I, *Hae*III, *Hin*fI, *Mbo*I, *Mbo*II, *Nci*I, and *Rsa*I) were then selected to perform the complete analysis, on the basis of their polymorphism and the number of restriction sites observed. As both amplified mtDNA segments were adjacent, restriction digests were made on pooled aliquots of 2–10 µL each, with conditions recommended by suppliers (Gibco BRL, Pharmacia, Promega). Resulting fragments were electrophoretically separated on 1.2% agarose gels, ethidium bromide stained, and photographed under ultraviolet light. A 20-µL mixture of lambda-DNA cut with *Hin*dIII and lambda-DNA double digested with *Eco*RI and *Hin*dIII was used as a size standard.

Distinct single restriction fragment patterns were identified by a specific letter in order of appearance. As changes in mtDNA fragment patterns could be accounted for by specific restriction gains or losses, a matrix of restriction sites presence–absence was resolved for each species from mtDNA fragment patterns (e.g., Bernatchez and Dodson 1991). Thus, each fish was assigned a composite mtDNA genotype representing distinct combinations of polymorphic restriction sites observed across 10 restriction enzymes. The overall diversity of mtDNA genotypes within each species was quantified by the nucleon (or haplotype) diversity (*h*) and nucleotide diversity (*p*) indices of Nei and Tajima (1981).

The mtDNA data were analysed by both distance- and character-based methods. The purpose of the distance-based analysis was to compare the extent of mean sequence divergence among mtDNA genotypes observed among the four species whereas character-based analysis was performed to delineate significant phylogenetic groupings within each species. No attempt was made to assess interspecific relationships, as restriction fragment patterns were too divergent to infer restriction site differences among species. Nucleotide sequence divergence among mtDNA genotypes (*p*; Nei and Li 1979) was estimated for each species using program D of the REAP software package (McElroy et al. 1992). The resulting distance matrices were used to build phenograms with the program NEIGHBOR (option UPGMA) of the PHYLIP 3.5c computer package (Felsenstein 1993). The UPGMA method was preferred to permit mtDNA sequence divergence levels to be easily compared across the four species, and to facilitate comparisons with the large number of UPGMA-based mtDNA trees in the literature. Phylogenetic relationships among mtDNA genotypes were assessed individually for each species using the restriction site presence–absence matrix to generate phylogenetic trees according to Wagner parsimony criteria using the MIX program (PHYLIP 3.5c). Majority-rule consensus trees were constructed using the CONSENSE program and confidence statements on branches were estimated by running the MIX program on 100 bootstrapped matrices of restriction site data generated by the SEQBOOT program.

The null hypothesis that each species is composed of a single gene pool was tested by first analysing the geographical heterogeneity of mtDNA genotypes among sites using $\chi^2$ randomization tests (Roff and Bentzen 1989) with 1000 permutations performed by the MONTE program of REAP. The extent of geographic differentiation of mtDNA diversity was also assessed by quantifying the interpopulation components of molecular variance ($Phi_{ST}$) using the program AMOVA (Excoffier et al. 1992). This estimate takes into account both the genotype frequency distribution and the number of restriction-site differences between them as a Euclidian distance measure. The statistical significance of $Phi_{ST}$ values was tested using a random permutation procedure available in AMOVA. Fixation indices ($F_{ST}$; Wright 1978) were also computed with AMOVA by omitting the molecular information of divergence among mtDNA genotypes and considering those as equidistant alleles of a single locus (Excoffier et al. 1992). The amount of gene flow within each species was quantified by the effective number of females exchanged per generation according to the approximation $N_e m_f = (1/ F_{ST} - 1)/2$. Although the veracity of the absolute $N_e m_f$ estimates depends on several assumptions that may not be met in the present situation (e.g., $m$ <1, population equilibrium with respect to genetic drift and migration, selective neutrality, island model of population structure), they nevertheless provide a comparative basis for estimating differential gene flow among the four species. Finally, we also computed a localization index that expresses the proportion of mtDNA genotypes observed in multiple individuals, yet confined to a single collection locale (Avise 1992).

## Results

### mtDNA diversity

The 10 restriction enzymes used generated a total of 246 restriction sites (restriction site patterns available from the authors) that resolved 103 site patterns and allowed the definition of 81 mtDNA genotypes across the four species analysed (Table 1). The number of mtDNA genotypes detected was highly variable among species, with only 7 in grass carp and up to 28 in silver carp (Table 2). Consequently, their estimates of overall nucleon and nucleotide diversity were also very different (Table 2). Nucleon diversity index was high in black carp (0.89), intermediate in silver and bighead carp (0.681 and 0.584), and very low in grass carp (0.231). Silver carp had the highest nucleotide diversity. Black and bighead carp showed intermediate, yet relatively high, values of nucleotide diversity whereas that observed in grass carp was very low.

Contrasting patterns of mtDNA diversity among species were also illustrated by phenograms clustering genotypes according to matrices of sequence divergence estimates (Fig. 2), and majority-rule consensus trees relating genotypes from the presence–absence of synapomorphic restriction sites (Fig. 3). UPGMA phenograms obtained for black and bighead carp showed similar topology and length (Figs. 2*b* and 2*d*). Thus, the mean sequence divergence estimates of their deepest branch lengths were almost identical ($p$ = 0.0079 in bighead and 0.0075 in black carp), and no major groupings based on divergence discontinuity were observed in the two phenograms. The lack of major phylogenetic subdivisions within these two species was also confirmed by the consensus trees

**Table 1.** Definition and absolute abundance of composite mtDNA genotypes resolved by PCR-RFLP analysis of ND5–ND6 and cytochrome *b* control-region segments for four carp species from the Yangtze River.

| Genotype | *Alu*I | *Ava*II | *Dde*I | *Hae*II | *Hha*I | *Hin*fI | *Mbo*I | *Mbo*II | *Nci*I | *Rsa*I | *N* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Silver carp** | | | | | | | | | | | |
| SC1 | A | A | A | A | B | A | B | D | A | A | 1 |
| SC2 | A | A | A | B | A | B | A | E | A | A | 2 |
| SC3 | A | A | B | A | B | A | A | B | A | A | 1 |
| SC4 | B | B | C | C | B | C | B | C | A | D | 1 |
| SC5 | A | A | B | A | B | A | A | D | A | A | 49 |
| SC6 | B | D | C | G | A | D | A | C | A | B | 1 |
| SC7 | B | D | C | G | B | D | A | C | A | A | 1 |
| SC8 | B | A | A | B | A | B | B | A | B | A | 1 |
| SC9 | A | A | B | E | B | A | A | D | A | A | 2 |
| SC10 | B | A | A | B | A | B | A | E | B | C | 1 |
| SC11 | C | A | A | B | A | E | B | A | B | C | 1 |
| SC12 | A | A | B | A | B | A | B | D | A | A | 2 |
| SC13 | B | C | C | G | B | F | A | E | A | D | 2 |
| SC14 | A | A | B | A | B | F | A | E | A | A | 1 |
| SC15 | B | C | C | D | B | F | A | D | A | D | 1 |
| SC16 | B | A | A | B | A | B | B | E | B | C | 1 |
| SC17 | A | A | B | A | B | A | A | D | A | C | 3 |
| SC18 | A | A | B | E | B | A | A | D | C | A | 3 |
| SC19 | B | C | D | F | B | F | A | D | A | D | 1 |
| SC20 | A | A | B | E | B | A | A | D | C | C | 6 |
| SC21 | B | C | D | G | B | F | A | D | A | D | 1 |
| SC22 | A | C | E | A | B | F | A | D | A | C | 1 |
| SC23 | A | C | E | A | B | A | A | D | A | C | 4 |
| SC24 | B | A | D | F | B | A | A | D | A | D | 1 |
| SC25 | A | C | B | A | B | A | A | D | A | C | 1 |
| SC26 | B | A | D | F | B | F | A | D | A | B | 1 |
| SC27 | A | C | E | A | B | A | A | D | A | A | 1 |
| SC28 | A | C | D | G | B | A | A | D | A | D | 1 |
| **Bighead carp** | | | | | | | | | | | |
| BH1 | A | A | C | A | A | A | A | A | A | A | 8 |
| BH2 | A | A | A | A | B | C | B | A | A | B | 2 |
| BH3 | A | A | B | A | B | A | C | A | A | B | 8 |
| BH4 | A | A | B | A | B | B | A | A | A | B | 1 |
| BH5 | A | A | A | A | B | C | A | A | A | B | 1 |
| BH6 | A | A | B | A | B | C | C | A | A | B | 39 |
| BH7 | A | A | C | A | B | A | D | A | A | A | 3 |
| BH8 | A | A | B | A | B | D | B | A | A | B | 3 |
| BH9 | A | A | B | A | B | C | A | A | A | A | 1 |
| BH10 | A | A | B | A | B | C | C | A | A | A | 1 |
| BH11 | A | A | B | A | B | D | C | A | A | B | 10 |
| BH12 | A | A | B | A | B | B | C | A | A | B | 3 |
| BH13 | A | A | C | A | A | D | C | A | A | B | 1 |
| BH14 | A | A | B | A | B | A | B | A | A | B | 2 |
| BH15 | A | B | B | A | B | C | C | A | A | B | 1 |
| BH16 | A | A | B | A | B | B | B | A | A | B | 2 |
| BH17 | A | A | C | A | B | B | B | A | A | A | 1 |
| BH18 | A | A | B | A | A | D | B | A | A | B | 2 |
| BH19 | A | A | C | A | B | D | B | A | A | A | 1 |
| **Grass carp** | | | | | | | | | | | |
| GC1 | A | A | A | B | B | A | A | A | A | A | 3 |
| GC2 | A | A | A | A | A | A | A | A | A | A | 79 |
| GC3 | A | A | A | C | A | A | A | A | A | A | 1 |
| GC4 | A | A | A | A | A | B | A | A | A | A | 1 |
| GC5 | A | B | A | A | A | A | A | A | A | A | 4 |
| GC6 | A | A | B | A | A | A | A | A | A | A | 1 |
| GC7 | A | A | A | A | B | A | A | A | A | A | 1 |

**Table 1** (*concluded*).

| Genotype | *Alu*I | *Ava*II | *Dde*I | *Hae*II | *Hha*I | *Hin*fI | *Mbo*I | *Mbo*II | *Nci*I | *Rsa*I | *N* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | **Black carp** | | | | | | |
| BC1 | A | A | A | B | A | B | A | A | A | A | 2 |
| BC2 | B | A | A | A | A | B | A | A | A | A | 2 |
| BC3 | B | A | A | C | A | B | B | A | A | A | 11 |
| BC4 | A | A | A | B | A | C | B | A | A | A | 16 |
| BC5 | A | A | A | B | A | B | B | A | A | A | 1 |
| BC6 | B | A | A | A | A | C | B | A | A | A | 8 |
| BC7 | A | A | A | C | A | D | B | A | A | A | 7 |
| BC8 | B | A | A | A | A | C | A | A | A | A | 13 |
| BC9 | A | A | A | C | A | A | A | C | A | A | 9 |
| BC10 | A | A | A | C | A | A | A | A | A | A | 2 |
| BC11 | A | A | A | B | A | A | B | A | A | A | 2 |
| BC12 | A | A | A | A | A | C | C | A | A | A | 1 |
| BC13 | C | A | A | A | B | B | B | A | A | A | 1 |
| BC14 | B | A | A | A | A | C | B | A | A | A | 5 |
| BC15 | B | A | A | A | B | B | B | A | A | A | 1 |
| BC16 | D | A | A | B | A | A | B | A | A | A | 1 |
| BC17 | D | A | A | C | A | C | B | A | A | A | 1 |
| BC18 | B | A | A | B | A | D | B | A | A | A | 1 |
| BC19 | A | A | A | A | A | A | B | A | A | A | 1 |
| BC20 | C | A | A | A | A | D | B | A | A | A | 1 |
| BC21 | B | A | A | A | A | E | A | A | A | A | 1 |
| BC22 | A | A | A | C | A | C | A | C | A | A | 1 |
| BC23 | A | A | A | D | A | C | B | A | A | A | 1 |
| BC24 | A | A | A | A | A | D | B | B | A | A | 1 |
| BC25 | B | A | A | C | A | A | A | C | A | A | 1 |
| BC26 | A | A | A | A | A | A | A | A | A | A | 1 |
| BC27 | B | A | A | A | A | A | A | C | A | A | 1 |

**Table 2.** Sample size for three locations, number of mitochondrial genotypes, overall nucleon diversity (*h*), and nucleotide diversity (*p*) for four carp species from the Yangtze River.

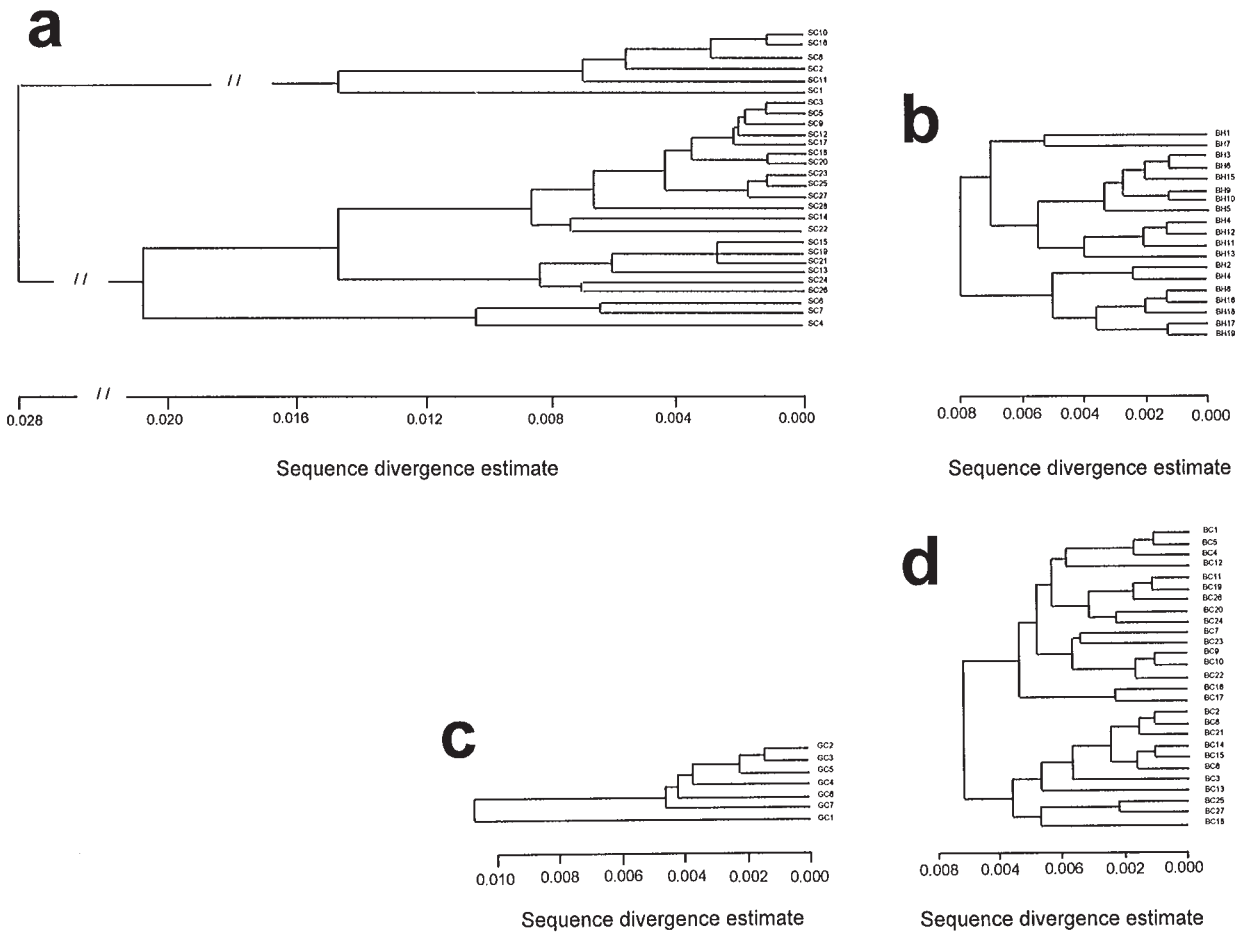
| | Sample size | | | No. of haplotypes | Nucleon diversity | Nucleotide diversity |
|---|---|---|---|---|---|---|
| | SO | RC | WH | | | |
| *Hypophthalmichthys molitrix* (silver carp) | 30 | 31 | 31 | 28 | 0.681 | 0.018 |
| *Aristichthys nobilis* (bighead carp) | 30 | 30 | 30 | 19 | 0.584 | 0.008 |
| *Ctenopharyngodon piceus* (grass carp) | 30 | 30 | 30 | 7 | 0.231 | 0.002 |
| *Mylopharyngodon piceus* (black carp) | 31 | 30 | 32 | 27 | 0.890 | 0.011 |

**Note**: Sampling locations are shown in Fig. 1. SO, Swan Oxbow; RC, Ruichang; WH, Wuhu.

in which only one and two pairs of genotypes clustered together in more than 50% of the bootstrap replicates for the bighead and black carp, respectively (Figs. 3*b* and 3*d*). Although a much lower number of genotypes was observed in grass carp, its deepest branch length was higher than for those of black and bighead carp ($p = 0.0103$) and a major divergence discontinuity was observed between genotype GC1 and all others that comprised a single grouping (Figs. 2*c* and 3*c*). The phylogenetic distinction between GC1 and other genotypes was supported in 100% of the bootstrap replicates. The depth of the UPGMA phenogram was much more pronounced in silver carp, and its topology much more structured than that of the other three species. Thus, genotypes first clustered into two major assemblages between which mean sequence divergence was high ($p = 0.028$) (Fig. 2*a*). This dichotomy was also supported in 100% of the bootstrap replicates (Fig. 3*a*). Additional groupings based on sequence divergence discontinuities and bootstrap values were also observed, namely between SC1 and other genotypes within the upper major grouping. Within the second major grouping, genotypes SC4, SC6, and SC7 formed a significant cluster (percent bootstrap value = 84%) that diverged from others by a mean sequence divergence of 0.022. An additional subdivision within the second major grouping was also observed at a mean sequence divergence of 0.015 that separated genotypes SC15, SC19, SC21, SC13, SC24, and SC26 from all remaining ones (Fig. 2*a*). However, the topology of this latter branching pattern differed in the consensus tree (Fig. 3*a*). Thus, the cluster SC15-SC26 was not supported by the bootstrap analysis. Instead, these genotypes, together with genotypes SC4, SC6, and SC7, formed a group supported at 64% bootstrap level that clustered distinctively from the remaining genotypes.

Differences ($P < 0.001$) in the frequency distribution of mtDNA genotypes among sampling sites were observed in

**Fig. 2**. UPGMA phenograms clustering mtDNA composite genotypes of four carp species from the Yangtze River according to the distance matrix of sequence divergence estimates: (*a*) silver carp, (*b*) bighead carp, (*c*) grass carp, (*d*) black carp. The mtDNA genotypes are defined in Table 2.

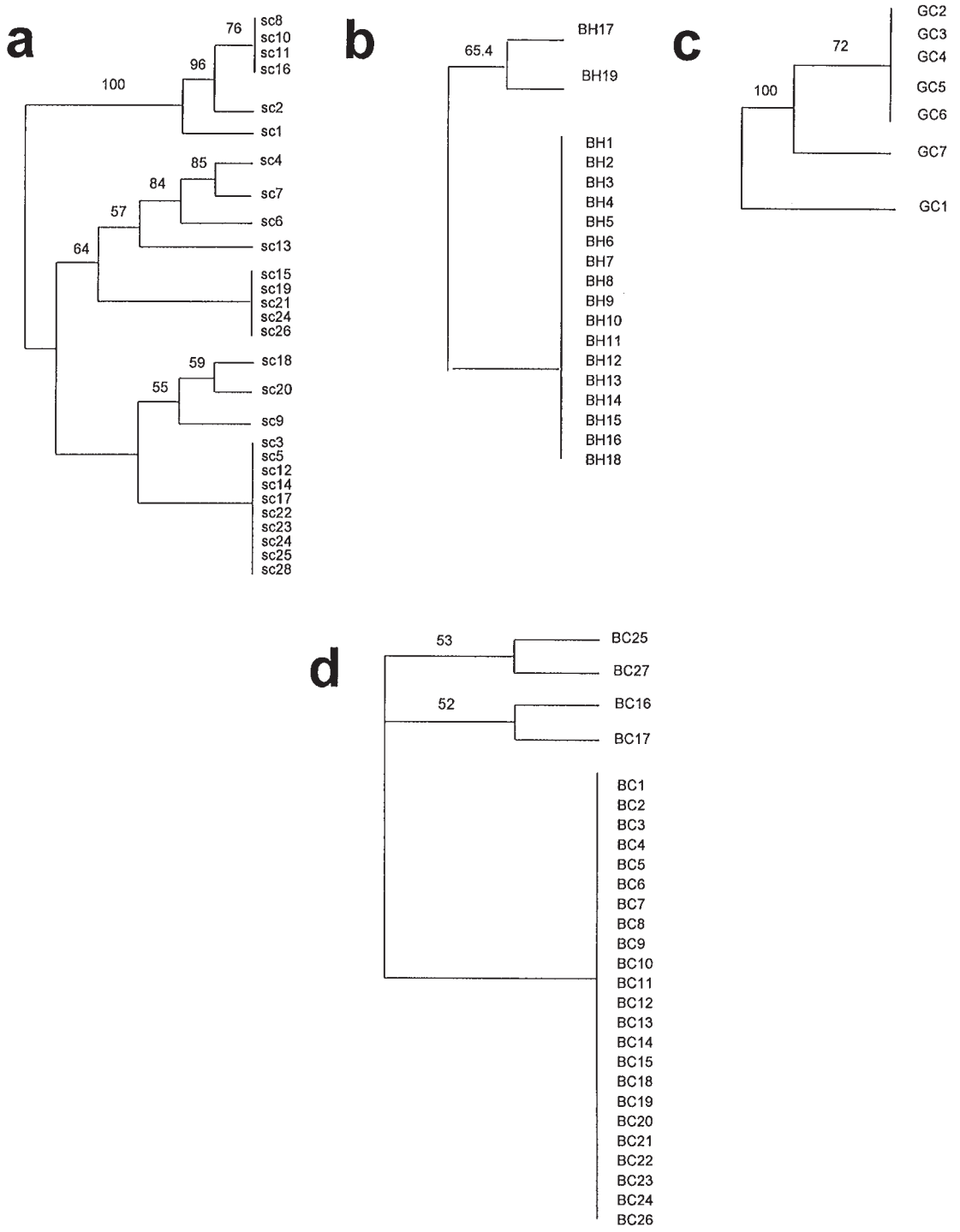



bighead, black, and silver carp, indicating that these were composed of more than one genetic stock (Tables 3 and 4). Pairwise $\chi^2$ tests between sites for silver and bighead carp ($P < 0.001$) (data not shown) provided further evidence that fish from different nursery grounds belonged to genetically distinct populations. This was also suggested by the high numbers of private genotypes unique to each sampling site for both species. Thus, 12, 5, and 7 private genotypes were observed at the midstream (Swan Oxbow), mid-downstream (Ruichang), and downstream (Wuhu) sites, respectively, in silver carp. For bighead carp, eight private genotypes were observed at both the midstream and downstream sites whereas the mid-downstream site was fixed for the single genotype BH6. For black carp, the mid-downstream site (Ruichang) was highly distinct ($P < 0.001$) from either the midstream (Swan Oxbow) or the downstream site (Wuhu) whereas no significant difference was observed between these latter two. Genetic distinctiveness among locations was also supported by the occurrence of private genotypes at the midstream, mid-downstream, and downstream sites (four, seven, and four, respectively). Contrary to the other three species, the frequency distribution of mtDNA genotypes was very homogeneous in grass carp ($P = 0.432$), thus providing no evidence for the existence of more than one genetic stock. The high and moderate localization index estimates for silver carp (0.78) , bighead carp (0.73), and black carp (0.33) also reflected the genetic differentiation among sites for these species, whereas that of grass carp was zero (0.00).

Fixation indices allowed further assessment of the extent of genetic heterogeneity and amount of gene flow among sites for each species (Table 4). $F_{ST}$ values resulting from the nested analysis of population structure revealed that bighead carp was by far the most highly structured species with 32.7% of the total diversity explained by intersite variance. This suggested a relatively low level of effective migration rate along the river, as reflected by a $N_e m_f$ value of 1.03. High, although less pronounced, interpopulation diversity was also observed in silver and black carp ($P < 0.001$), which suggested higher, yet restricted gene flow among sites for these two species ($N_e m_f = 8.59$ and 10.61). The absence of significant heterogeneity in the frequency distribution of grass carp genotypes was indicated by the low $F_{ST}$ and the highest estimate of gene flow ($N_e m_f = 35.21$) among sites.

Although the two parameters are highly correlated, the computation of interpopulation molecular variance ( $Phi_{ST}$) is complementary to that of $F_{ST}$ because it accounts for nucleotide divergence among genotypes as well as their frequency distribution. Consequently, comparisons of the two values allowed the assessment of a possible phylogenetic (historical)

**Fig. 3.** Majority-rule consensus trees relating mtDNA composite genotypes of four carp species from the Yangtze River: (*a*) silver carp, (*b*) bighead carp, (*c*) grass carp, (*d*) black carp. Bootstrap estimates (in percentages) that were higher than 50% are indicated above branches. All mtDNA genotypes with branching patterns supported at less than 50% were collapsed together. The mtDNA genotypes are defined in Table 2.




component to the pattern of interpopulation genetic diversity (Bernatchez and Martin 1996). Thus, estimates obtained for the two values were comparable in bighead carp ( $Phi_{ST}$ = 0.253, $F_{ST}$ = 0.327), which suggested that no phylogenetic component accounts for the population structure observed in that species. Indeed, the extent of population differentiation was largely due to the fixation of one genotype (BH6) at the midstream site and the alternative occurrence of many private ones at other sites, without any association with their phylogenetic relationships (Table 3, Figs. 2*b* and 3*b*). In contrast, the silver carp $Phi_{ST}$ estimate was approximately twice that of $F_{ST}$ (0.103 versus 0.055), suggestive of an interpopulation

**Table 3.** Frequency distribution of mtDNA genotypes (Gen.) among three sampling sites for four carp species from the Yangtze River.

| Silver carp | | | | Bighead carp | | | | Grass carp | | | | Black carp | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Gen. | SO | RC | WH | Gen. | SO | RC | WH | Gen. | SO | RC | WH | Gen. | SO | RC | WH |
| SC1 | 1 | 0 | 0 | BH1 | 8 | 0 | 0 | GC1 | 2 | 1 | 0 | BC1 | 2 | 0 | 0 |
| SC2 | 2 | 0 | 0 | BH2 | 2 | 0 | 0 | GC2 | 26 | 26 | 27 | BC2 | 2 | 0 | 0 |
| SC3 | 1 | 0 | 0 | BH3 | 1 | 0 | 7 | GC3 | 1 | 0 | 0 | BC3 | 5 | 0 | 6 |
| SC4 | 1 | 0 | 0 | BH4 | 1 | 0 | 0 | GC4 | 1 | 0 | 0 | BC4 | 6 | 3 | 7 |
| SC5 | 14 | 23 | 12 | BH5 | 1 | 0 | 0 | GC5 | 0 | 2 | 2 | BC5 | 1 | 0 | 0 |
| SC6 | 1 | 0 | 0 | BH6 | 4 | 30 | 5 | GC6 | 0 | 1 | 0 | BC6 | 2 | 4 | 2 |
| SC7 | 1 | 0 | 0 | BH7 | 3 | 0 | 0 | GC7 | 0 | 0 | 1 | BC7 | 5 | 0 | 2 |
| SC8 | 1 | 0 | 0 | BH8 | 3 | 0 | 0 | | | | | BC8 | 3 | 8 | 2 |
| SC9 | 1 | 0 | 1 | BH9 | 1 | 0 | 0 | | | | | BC9 | 1 | 0 | 8 |
| SC10 | 1 | 0 | 0 | BH10 | 1 | 0 | 0 | | | | | BC10 | 1 | 0 | 1 |
| SC11 | 1 | 0 | 0 | BH11 | 4 | 0 | 6 | | | | | BC11 | 1 | 1 | 0 |
| SC12 | 2 | 0 | 0 | BH12 | 1 | 0 | 2 | | | | | BC12 | 1 | 0 | 0 |
| SC13 | 2 | 0 | 0 | BH13 | 0 | 0 | 1 | | | | | BC13 | 1 | 0 | 0 |
| SC14 | 1 | 0 | 0 | BH14 | 0 | 0 | 2 | | | | | BC14 | 0 | 5 | 0 |
| SC15 | 0 | 1 | 0 | BH15 | 0 | 0 | 1 | | | | | BC15 | 0 | 1 | 0 |
| SC16 | 0 | 1 | 0 | BH16 | 0 | 0 | 2 | | | | | BC16 | 0 | 1 | 0 |
| SC17 | 0 | 3 | 0 | BH17 | 0 | 0 | 1 | | | | | BC17 | 0 | 1 | 0 |
| SC18 | 0 | 1 | 2 | BH18 | 0 | 0 | 2 | | | | | BC18 | 0 | 1 | 0 |
| SC19 | 0 | 1 | 0 | BH19 | 0 | 0 | 1 | | | | | BC19 | 0 | 1 | 0 |
| SC20 | 0 | 1 | 5 | | | | | | | | | BC20 | 0 | 1 | 0 |
| SC21 | 0 | 1 | 0 | | | | | | | | | BC21 | 0 | 1 | 0 |
| SC22 | 0 | 0 | 1 | | | | | | | | | BC22 | 0 | 1 | 0 |
| SC23 | 0 | 0 | 4 | | | | | | | | | BC23 | 0 | 0 | 1 |
| SC24 | 0 | 0 | 1 | | | | | | | | | BC24 | 0 | 0 | 1 |
| SC25 | 0 | 0 | 1 | | | | | | | | | BC25 | 0 | 0 | 1 |
| SC26 | 0 | 0 | 1 | | | | | | | | | BC26 | 0 | 1 | 0 |
| SC27 | 0 | 0 | 1 | | | | | | | | | BC27 | 0 | 0 | 1 |
| SC28 | 0 | 0 | 1 | | | | | | | | | | | | |

**Note**: Sampling locations are shown in Fig. 1. SO, Swan Oxbow; RC, Ruichang; WH, Wuhu.

**Table 4.** $\chi^2$, $\text{Phi}_{ST}$, $F_{ST,}$, and $N_e m_f$ values and their associated probabilities for four carp species from the Yangtze River.

| | $\chi^2$ | $P$ | $F_{ST}$ | $P$ | $\text{Phi}_{ST}$ | $P$ | $N_e m_f$ |
|---|---|---|---|---|---|---|---|
| Silver carp | 76.37 | <0.001 | 0.055 | 0.001 | 0.103 | 0.002 | 8.59 |
| Bighead carp | 111.73 | <0.001 | 0.327 | 0.001 | 0.253 | 0.001 | 1.03 |
| Grass carp | 12.04 | 0.432 | 0.014 | 0.723 | 0.002 | 0.456 | 35.21 |
| Black carp | 83.51 | <0.001 | 0.045 | 0.001 | 0.109 | 0.001 | 10.61 |

phylogenetic component. Indeed, five out of six genotypes belonging to the most divergent phylogenetic grouping (SC1, SC2, SC8, SC10, SC11) in that species, as well as all genotypes comprising the second most divergent group (SC4, SC6, SC7), were confined to the upstream location (Table 3, Figs. 2*a* and 3*a*). In black carp, the $\text{Phi}_{ST}$ estimate was also about twice that of $F_{ST}$ (0.109 versus 0.045), although no significant geographic pattern of related genotypes could be depicted owing to the poor phylogenetic resolution of mtDNA genotypes in that species (Fig. 3*d*). As for the $F_{ST}$ estimate, the $\text{Phi}_{ST}$ value was very low in grass carp, in accordance with its lack of population differentiation.

## Discussion

### mtDNA diversity in Chinese carps

The PCR-RFLP analysis of the mtDNA segment encompassing the ND5 and ND6 subunits of the NADH dehydrogenase, and the cytochrome *b* genes, and the control region revealed considerable polymorphism in silver, bighead, and black carp, as exemplified by the high numbers of genotypes detected, as well as nucleon and nucleotide diversity values. The extent of their mtDNA diversity can be further appreciated by comparison with those reported among other fishes. Thus, levels of mtDNA intraspecific sequence divergence observed for these three species of carps in the Yangtze River alone (average $p$ ranging from 0.008 to 0.018) were higher than those reported for most marine, anadromous, and freshwater fishes of northern latitudes for which average intraspecific sequence divergence typically varies between 0.002 and 0.018, including over the entire species distribution range (Billington and Hebert 1991; L. Bernatchez, unpublished data). Sequence divergence estimates observed within these species were surpassed only by those reported among geographic discontinuities of major phylogenetic groupings within freshwater species whose distribution ranges were not covered by Pleistocene glaciation events (e.g., Bermingham and Avise 1986; Ovenden et al. 1988; Fajen and Breden 1992; Dodson et al. 1995).

In contrast with the other three species, extremely reduced mtDNA diversity was observed in grass carp. Many factors can potentially be responsible for this. Globally, differences in mtDNA diversity could signal either departures from neutrality

and (or) differential mutation rate, as well as differences in effective population size. It is recognized that mtDNA is not a strictly neutral marker, and in fact, evidence for differential selection among mtDNA genotypes is being increasingly reported (reviewed in Ballard and Kreitman 1995). Even though one cannot rule out the possibility that departures from neutrality can vary among species, it remains to be demonstrated how this could generate differential patterns of mtDNA diversity as observed here among closely related species found in the same environment. Similarly, the possibility of differential mtDNA mutation rate cannot be strictly eliminated but seems unlikely for several reasons. It has been documented that a given segment of the mitochondrial genome can evolve at a different rate in different organisms (e.g., Brown et al. 1979; Thomas and Beckenbach 1989; Avise 1992; Avise et al. 1992; Martin et al. 1992; Martin and Palumbi 1993; Bentzen et al. 1993). Among the main factors thought to be responsible for variation in mutation rates are interspecific differences in metabolic rate, thermal habit, body size, and generation time (reviewed in Rand 1994). None of these factors appears to vary sufficiently between grass and other carp species to explain the magnitude of their discrepancy in mtDNA diversity. All four species are poikilotherms, they are found in the same thermal habitat, and they have similar generation times (Hydrobiology Research Institute 1976).

The possibility that differences in effective population size may account for interspecific differences in the magnitude of mtDNA variation is equally problematic. Effective population size, which can be defined instantaneously as the effective number of individuals contributing to the next generation, will depend on adult population size, sex ratio, overlap in generations, as well as variance in reproductive success, which will largely depend on fecundity (Avise et al. 1984, 1987; Hedgecock 1994). Current population size estimates indicate that grass carp is the most abundant species in the Yangtze River, being approximately 10 times more numerous than silver and bighead carp, which exhibited much higher mtDNA diversity. There are no striking differences in sex ratio and in the number of overlapping generations among species, all of them being multiple spawners that can reproduce yearly from age 4 (silver carp), 5 (bighead, grass carp), or 7 (black carp) and up to age 20. Average female fecundity is comparable among species, varying between $7 \times 10^5$ and $12 \times 10^5$ eggs. Altogether, these observations suggest that differences in mtDNA diversity observed among carp species in the Yangtze River are poorly correlated with their current effective population sizes.

Alternatively, the effective population size can be calculated over generations as the harmonic mean of effective population sizes at each generation (Hartl and Clark 1989). There is sound evidence that, under the inbreeding theory applied to neutral alleles, the long-term effective population size is vastly smaller than current abundance for many species (e.g., Nei and Graur 1984; Avise 1992). The lower mtDNA diversity observed in grass carp is consistent with the hypothesis that historical demographic influences, such as proportional fluctuations in female population size, perhaps through repeated bottlenecks, have been more important in that species than the other three. A better understanding of their historical demography could be obtained by performing a more comprehensive phylogeographic study of these species (e.g., Avise 1992; Bernatchez and Dodson 1994).

**Carp population structure in the Yangtze River**

The statistical analysis of mtDNA genotype frequency distribution, estimates of molecular variance, fixation, and localization indices all revealed significant genetic differences among juvenile fish from different nursery areas for three of the four species. These results indicate that the species are each composed of more than one genetic stock within the Yangtze River. Therefore, the mtDNA data contrast with a previous study of enzymatic loci variation performed on the same specimens, which revealed no significant differences in allele frequency among sites, and consequently did not allow rejection of the null hypothesis that these species are each composed of a single gene pool (Zhao and Li 1995). Among the many factors that could be responsible for this, the most likely is the lack of allozyme polymorphism that was detected in allozymes, which precluded meaningful statistical assessments of genetic diversity (Zhao and Li 1995). A better resolution of fish population structure by mtDNA analysis compared with allozymes has been reported in many cases (reviewed in Ward and Grewe 1994). However, this may not always be the case, and in fact the reverse situation has also been reported in several instances (e.g., Ferguson et al. 1991). This was also illustrated by the reduced mtDNA polymorphism observed in grass carp, which precluded any assessment of population structure, as for allozymes. In such a case, the use of potentially finer analytical tools, such as the analysis of microsatellite loci, may prove more useful than mtDNA to infer genetic diversity (e.g., Angers et al. 1995).

Although silver, bighead, and black carp all showed genetic heterogeneity among nursery areas, they exhibited several differences in their patterns of genetic diversity. The genetic distinction among nursery grounds within black and bighead carp was primarily based on the frequency distribution of mtDNA genotypes, regardless of their relatedness. In contrast, the distinctiveness of the midstream nursery ground in silver carp was associated with the private occurrence of genotypes comprising a phylogenetically distinct mtDNA group that diverged from other genotypes by 2.8% sequence divergence on average. This suggests that unlike the other three species, two ancestral populations of silver carp that evolved in allopatry colonized the Yangtze River (e.g., Avise et al. 1987), one of which remained confined to the upper reaches of the river. A more complete phylogeographic study of the species would allow a firmer assessment of this hypothesis.

Bighead carp was characterized by overall higher $F_{ST}$ and $\text{Phi}_{ST}$ estimates than the other species. These values were most likely inflated by the extremely reduced mtDNA polymorphism at the mid-downstream station (Ruichang) where fish were fixed for a single genotype. In fact, the estimations of $F_{ST}$ and $\text{Phi}_{ST}$ were very similar to those obtained for silver and black carp when only the midstream and downstream sites were considered ($F_{ST} = 0.05$, $\text{Phi}_{ST} = 0.08$). No obvious reason beside historical population bottlenecks can explain the present mtDNA monomorphism observed for bighead carp in the mid-downstream section of the river.

In black carp, the mid-downstream site was highly distinct from either the midstream or the downstream site whereas no significant difference was observed between the midstream and downstream site. A plausible explanation for this may be that gene flow is restricted between the upstream and downstream sites, but insufficient time has passed since population

founding for equilibrium to have been reached with respect to genetic drift (e.g., Pogson et al. 1995).

### A possible mechanism for carp population structure in the Yangtze River

Despite the extensive literature on genetic population structure in fish (reviewed in Gyllenstein 1985; Ovenden 1990; Billington and Hebert 1991; Ward et al. 1994), relatively few studies have addressed the question of genetic variance partitioning within a river system. The majority of them deal with salmonids, with several exceptions (e.g., Brown et al. 1992; Ferguson et al. 1993). Generally speaking, the extent of population substructuring within a river has been implicitly attributed to the precision of homing (or the lack thereof) and (or) the existence of physical barriers to gene flow (e.g., Heggberget et al. 1986; Moran et al. 1995). However, no attempt has been made to provide a more theoretical framework that could relate patterns of within-river population diversity to deterministic causes.

In the past decade, the member–vagrant hypothesis has provided a major theoretical framework for studying the role of ecological processes in determining the spatial patterns of abundance in aquatic species, particularly for marine ecosystems (Sinclair 1988). This hypothesis proposes that population structure evolves primarily as a consequence of selective forces that maximize the probability of encounter among sexually mature individuals and the survival of young life-history stages. This infers that the number of populations comprising a given species is determined by the number of geographic settings within which the species life cycle is capable of closure, from hatching to spawning (Sinclair 1988).

Such geographic settings for carps can be inferred from their life history in the Yangtze River. Thus, the four species use the same spawning grounds that are discontinuously distributed from the upper to the lower reaches of the river. Larvae are passively transported downstream towards nursery areas that may be found in the river itself or in connecting lakes. In the latter case, young fry may actively swim to the lakes soon after metamorphosis. They feed and grow in nursery areas for 4–6 years until they reach sexual maturity and migrate back to spawning grounds. Thus, the geographic settings allowing closure of the life cycle of carp may correspond to a given section of the river that includes spawning grounds and the nearest downstream nursery grounds to which young life stages migrate and grow until reproductive age. Under the member–vagrant hypothesis, life cycle closure within such spatial constraints would be selected for, both by an increased survival of young fish adapted to remain in the closest nursery grounds compared with those dispersing further downstream, and by the increased fitness resulting from the reduced energetic cost of reproductive migration and increased probability of sexual encounter of those individuals found in relative proximity to the source of reproduction itself (Sinclair 1988). The fact that genetic distinctiveness was observed among fish from different nursery grounds suggests that the number of populations of silver, bighead, and black carp in the Yangtze River is determined by the number of such geographic settings within which their life cycle can be completed. This hypothesis is also indirectly supported by the fact that there are no apparent physical barriers to gene flow in the system and that we found no evidence for isolation by distance, thus suggesting that these factors are not important in structuring carp genetic diversity in the Yangtze River. Nevertheless, a firmer assessment of the hypothesis must await more detailed genetic analyses that should include individuals sampled at both spawning sites and nursery grounds, as well as additional spawning-area – nursery-ground settings found along the river.

### Implications for management and conservation

The demonstration that silver, bighead, and black carp consist of more than one genetic stock has several management implications. Optimal sustainable harvesting can only be achieved by managing genetically distinct stocks individually (Ryman et al. 1995). Thus far, management policies, such as setting harvest limits of both fry and older fish, have been designed under the premise that each carp species consists of a single population unit within the Yangtze River. Such policies may have several potential drawbacks. In particular, fixing harvest limits on the basis of the overall abundance in the system could lead to sequential recruitment failure from the smaller to the more important populations. In fact, the overall abundance of all carp species has declined dramatically over the past two decades in the Yangtze River (Yi et al. 1988; Li 1990; Lu and Li 1992). Although it would certainly not solve all problems related to carp exploitation, reorienting management policies on the principle that these species are composed of genetically distinct populations associated with different river sections is desirable.

The finding that carps comprise genetically distinct populations also has implications for the conservation of their genetic diversity. To preserve the four species in the face of their population declines, the Chinese government has recently undertaken the creation of genetic pools, located near Tongtinghu Lake. On the basis of the premise that each of the four species is composed of a unique, homogeneous genetic stock within the river system, these captive populations have been maintained by fish originating only from the middle reaches (Swan Oxbow area) of the Yangtze River. Although large captive populations may partly compensate for declines of wild populations, the actual conservation plan will only preserve a fraction of their overall genetic diversity within the river. It is thus advisable to found other captive genetic pools with fish originating from other sections of the Yangtze River, such as the Ruichang and Wuhu areas.

Finally, our results may change the vision of anticipated impacts of the Three Gorges Dam on the Yangtze River. There is little doubt that its ongoing construction near important spawning grounds will result in major losses of reproductive habitat in the middle reaches of the river. On the basis of the premise of a single population comprising each species, it may be anticipated that the resulting loss of recruitment in major upstream nursery grounds, such as Tongtinghu Lake, would rapidly be compensated by colonization of fish from other sections of the river. In contrast, evidence of population substructuring and low estimates of the numbers of migrants per generation between the midstream and more downstream sections of the river indicate that the rate of recolonization may be too slow to maintain sustainable levels of exploitation on the upstream nursery grounds.

In conclusion, our results illustrate the usefulness of mtDNA analysis to generate relevant information for the management and conservation of silver, bighead, and black carp.

In contrast, the lack of resolution in grass carp showed that this cannot be generalized to all species. Although this study was sufficient to reveal that, for three carp species, genetic diversity is not spatially homogeneous within the Yangtze River, it could not delineate exactly the number and distribution of distinct populations. We also recognize that the results obtained from a single locus must be interpreted cautiously. Clearly, further understanding of population structure and its consequences for carp management and conservation could be gained by analyses of rapidly evolving nuclear loci on fish from other sites along the river.

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