Preservation of the genetic diversity of a local common carp in the agricultural heritage rice–fish system

Weizheng Ren1,2, Liangliang Hu1,2, Liang Guo1,2, Jian Zhang3, Lu Tang3, Entao Zhang3, Jiaen Zhang3, Shiming Luo3, Jianjun Tang1,2, and Xin Chen1,2

1College of Life Sciences, Zhejiang University, Hangzhou 310058, China; and 2Department of Ecology, South China Agricultural University, Guangzhou 510642, China

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We examined how traditional farmers preserve the genetic diversity of a local common carp (Cyprinus carpio), which is locally referred to as “paddy field carp” (PF-carp), in a “globally important agricultural heritage system” (GIAHS), i.e., the 1,200-y-old rice–fish coculture system in Zhejiang Province, China. Our molecular and morphological analysis showed that the PF-carp has changed into a distinct local population with higher genetic diversity and diverse color types. Within this GIAHS region, PF-carp exist as a continuous metapopulation, although three genetic groups could be identified by microsatellite markers. Thousands of small farmer households interdependently obtained fry and parental carps for their own rice–fish production, resulting in a high gene flow and large numbers of parent carps distributing in a mosaic pattern in the region. Landscape genetic analysis indicated that farmers’ connectivity was one of the major factors that shaped this genetic pattern. Population viability analysis further revealed that the numbers of these interconnected small farmer households and their connection intensity affect the carps’ inherent genetic diversity. The practice of mixed culturing of carps with diverse color types helped to preserve a wide range of genetic resources in the paddy field. This widespread traditional practice increases fish yield and resource use, which, in turn, encourages farmers to continue their practice of selecting and conserving diverse color types of PF-carp. Our results suggested that traditional farmers secure the genetic diversity of PF-carp and its viability over generations in this region through interdependently incubating and mixed-culturing practices within the rice–fish system.

Significance

This paper contributes to understanding how traditional agriculture can maintain large genetic diversity. We quantify the effects of traditional farmer activities on the genetic diversity of indigenous common carp in the 1,200-y-old agricultural heritage rice–fish system. We show that small farmer households interdependently incubating fish fry for their rice–fish farming shape the genetic pattern and help to maintain high genetic diversity of this local common carp. We also show how the traditional practice of mixed culturing of diverse color types potentially promotes genetic diversity. We suggest that the locally adapted ways of traditional farmers obtaining and using local genetic resources for their farming play an important role in the biodiversity of farmed crops and animals. It can become a “hotspot” for genetic diversity conservation in agriculture.

Many traditional agricultural systems have been created, shaped, and maintained by generations of farmers and herdsmen who have used locally adapted practices to manage diverse natural resources (1). In addition to contributing to the food and livelihoods of people throughout the world, these traditional agricultural systems have maintained a rich diversity of genetic resources (e.g., local crop and animal varieties) (1–3). For example, a study of 27 crop species distributed in eight countries over the five continents demonstrated that such traditional agricultural systems maintain considerable crop genetic diversity (3). Similarly, many landraces of rice (4, 5), maize (6), sorghum (7, 8), manioc (9), fig (2), and indigenous animals (10, 11) have been preserved by traditional farmers. In addition to maintaining genetic resources, such traditional agricultural systems also serve as incubators for increased genetic diversity.

With the rapid development of modern agriculture and its reliance on high-yielding and genetically uniform varieties, many traditional agricultural systems have been progressively abandoned, resulting in the loss of local genetic resources and indigenous technical knowledge (1, 11–13). The genetic diversity of maize in Mexico, wheat in France, and rice in Bangladesh, for example, has greatly decreased over the last several decades (14, 15). About 20% of agricultural animals were listed as “at risk” by the Food and Agriculture Organization (FAO) (16, 17). Before more of the valuable knowledge and resources inherent in traditional agriculture are lost, it is essential that researchers now determine how traditional agricultural practices can preserve a high level of genetic diversities and how the practices and genetic diversity help traditional agriculture adapt to local conditions. Such studies should help in the design of genetic diversity preservation, and help in the design of sustainable agricultural systems that integrate traditional knowledge and modern technologies.

Several programs have been developed to preserve traditional agricultural systems and to rescue local agrobiodiversity and the associated indigenous wisdom (18). One of these programs, “Globally Important Agricultural Heritage Systems (GIAHS),” was initiated by the FAO, the United Nations Development Program, and the Global Environment Facility in 2002. Many important traditional agricultural systems worldwide have been identified and listed as GIAHS sites. The traditional rice–fish coculture system, which has been practiced by local farmers in the Zhejiang Province of China for more than 1,200 y (18), 19), was listed as one of the GIAHS sites in 2005 (www.fao.org/giahs/giahs-home/en/).

This traditional rice–fish coculture system is considered a sustainable form of agriculture that provides rice grain and fish for local farmers (19). It has also preserved both local rice varieties and indigenous common carp populations (20, 21). The indigenous carp, which is locally referred to as paddy field carp (PF-carp), is a local population of the common carp, Cyprinus carpio, an indigenous common carp in the 1,200-y-old agriculture heritage rice–fish system, which has been practiced by local farmers in the Zhejiang Province of China for more than 1,200 y (18, 19), was listed as one of the GIAHS sites in 2005 (www.fao.org/giahs/giahs-home/en/).

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1W.R., L.H., and L.G. contributed equally to this work.

2To whom correspondence may be addressed. Email: chen-tang@zju.edu.cn or chandt@zju.edu.cn.

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carpio. PF-carp has been cultured and passed down from generation to generation of rice–fish farmers (RF-farmers) in the GIAHS rice–fish culture system (22). How this indigenous carp is preserved in the rice–fish coculture system, however, has not been scientifically assessed.

In this study, we investigated how the genetic and phenotypic diversity of PF-carp have been maintained in the rice–fish coculture system. We also determine whether phenotypic diversity enhances the performance of PF-carp in rice fields. We conducted the study at the pilot site of the GIAHS rice–fish coculture system in Zhejiang Province, China (Fig. 1C and SI Appendix, Study Site and Rice–Fish Coculture System and Fig. S1).

Results

PF-Carp in the Rice–Fish System. With the mitochondrial D-loop gene sequences of PF-carp (determined in the current study) and five strains of common carp (obtained from GenBank), we developed a phylogenetic tree; the tree indicates substantial divergence between PF-carp and the other five strains of common carp that are widely cultured in non-rice field habitats in China (Fig. L4). A morphological cluster analysis based on eight traits (SI Appendix, Table S1) also indicated substantial differences between that PF-carp and these five common carp strains (Fig. L4).

The PF-carp in this GIAHS rice–fish system has evolved diverse body colors (Fig. 1B). A field survey was used to determine the spatial distribution of the main color types, which are red, red-black, and black (Fig. 1C). Landmark-based geometric morphometric analysis showed that these three color types significantly differed in body shape (SI Appendix, Fig. S2 and Table S2).

Genetic Diversity and Structure. Analysis of microsatellite markers revealed a high level of genetic diversity of PF-carp (SI Appendix, Table S3). Expected heterozygosity (He) and allele number (Na) of PF-carp at the village level were further compared with wild carp and cultured carp that live in non-rice field habitats (SI Appendix, Genetic Diversity Indices of Other Common Carp). For He, both the Kruskal–Wallis test and pairwise test showed that the He was significantly higher for PF-carp than for cultured carp (Kruskal–Wallis test: \( \chi^2 = 13.129, P = 0.0009 \); pairwise test: \( Z = -2.521, P = 0.012 \)), but did not significantly differ between PF-carp and wild carp (Kruskal–Wallis test: \( \chi^2 = 0.859, P = 1.062 \); pairwise test: \( Z = -0.944, P = 0.345 \)) (Fig. 2A and SI Appendix, Fig. S3A and Table S10). For Na, the Kruskal–Wallis test and pairwise test gave different results. The Kruskal–Wallis test showed that Na did not significantly differ between PF-carp and cultured carp (\( \chi^2 = 0.739, P = 1.1704 \)), but was significantly lower for PF-carp than for wild carp (\( \chi^2 = 10.382, P = 0.0038 \)) (Fig. 2B). The pairwise test showed that Na were significantly higher for PF-carp than for cultured carp (\( Z = -2.556, P = 0.011 \)), but did not significantly differ between PF-carp and wild carp (\( Z = -0.921, P = 0.357 \)) (SI Appendix, Fig. S3B and Table S10).

Analysis of molecular variance (AMOVA) was used to determine the percentage of genetic variation that was within populations of PF-carp (i.e., within a village), among populations (i.e., among villages), and among regions (i.e., among towns) in the study area. The results indicated that genetic variation was mainly within populations (93%), but was also significant among PF-carp populations (3%, \( P = 0.001 \)) and among regions (4%, \( P = 0.001 \)). Principal coordinate analysis (PCoA) indicated that there were no distinct genetic clusters (Fig. 2C).

Fig. 1. The PF-carp conserved in the GIAHS rice–fish coculture system. (A) Phylogenetic tree (red lines) and morphological clustering (blue lines) of PF-carp and other common carp; *, the recent name is Cyprinus rubrofuscus; †, the samples are from a cultured strain in this study. The name in the box refers to PF-carp, of which the major color types are shown in B. (B) The seven color types of the PF-carp. (C) Study area and geographical distribution of the color types. The maps at Top Left and Middle Left indicate the survey location in China and in Zhejiang Province, respectively. The bar chart at Bottom Left indicates the percentage of color types in the GIAHS area; “Others” include white, white with black spots, and jade with black spots. The map at Right indicates the distribution of the color types in the GIAHS area. Symbols indicate the relative abundance of each type at each location.
In a Bayesian procedure, samples of individual PF-carp were assigned to one of three genetic groups [SI Appendix, Assessment of the Number of Genetic Groups (K Value)]. All sampled locations (villages) showed high admixture rates of the groups. No distinct spatial distribution of the groups was found among the sampled geographic locations (Fig. 2D). Together with PCoA, the results indicated a continuous metapopulation of PF-carp in the GIAHS area.

Farmer Connectivity and Genetic Distance. Gene flow was indicated by the value of $N_{m}$ (number of migrants). $N_{m}$ values estimated by the Wright–Fisher model showed a high level of gene flow among PF-carp populations in different villages within the GIAHS area (Fig. 3A). The farmer survey also showed that there was a high rate of fish fry flow within the area (Fig. 3B). We reasoned that the flow of fish fry driven by farmer activities (e.g., parental carp exchange and fry transmission) promotes gene flow, resulting in a low genetic distance. We therefore assessed the correlation between farmer connectivity (mainly due to fry flow) and genetic distance by using cost distance modeling in a geographical information system (GIS). Farmer connectivity was evaluated by estimating the least-cost path (LCP; a low LCP value indicates high farmer connectivity) [SI Appendix, Estimation of the Least-Cost Path (LCP)].

The Mantel test showed that both the LCP matrix and geographic distance (GD) matrix were positively correlated with the genetic distance (Nei’s) matrix (Fig. 3C). A partial Mantel analysis that excluded the effect of GD still showed a positive correlation between genetic distance and LCP ($r_{partial} = 0.225$).

![Fig. 2. Genetic diversity and genetic structure of PF-carp based on microsatellite markers. (A and B) Expected He and Na in PF-carp at village level, in wild carp, or in cultured carp on non-rice field habitats (SI Appendix, Genetic Diversity Indices of Other Common Carp). Means with the same lowercase letters are not significantly different ($P > 0.05$). (C) PCoA of PF-carp populations from counties based on genetic distance (Nei’s). QT, Qingtian County; RA, Ruian County; Y1, Yongjia County. (D) Map of the genetic structure (the relative abundances of genetic groups 1, 2, and 3) of the PF-carp at the 33 sampled villages based on Bayesian analysis. BL, Bi-Lian town; DRY, Da-Ruo-Yan town; FL, Feng-Ling town; FS, Fang-Shan town; GA, Gui-Ao; JC, Jin-Chuan town; JP, Ju-Pu town; JZ, Ji-Zhai town; MA, Ming-Ao town; RZ, Ren-Zhuang town; SX, Shi-Xi town; WX, Wen-Xin town; XZS, Xiao-Zhou-Shan town; and ZD, Zhang-Dan town.](image)

![Fig. 3. Flows of gene and fry in the GIAHS area and the effects of GD and farmer connectivity on genetic distance. (A) Frequency spectra of $N_{m}$ values indicating gene flow. (B) Fry flow rate in the GIAHS area. (C) Correlations between genetic distance and GD, and with LCP. Genetic distance (Nei’s) was estimated based on Nei’s calculation (40). The LCP, which was used to evaluate farmer connectivity, was estimated by cost distance modeling with a GIS [see SI Appendix, Estimation of the Least-Cost Path (LCP)]. In this estimation, a low LCP value indicates high farmer connectivity.](image)
Farmer Connections and Genetic Diversity Maintenance. A survey of the farmers in the 33 sampled villages showed that there were three types of RF-farmers with respect to the maintenance of parental carp and the production of fry: those who have large numbers of parental carp and who provide fry to many other farmers; those who have small numbers of parental carp and who can obtain some of fry on site for themselves and neighboring farmers; and those who do not maintain parental carp and who must obtain fry from other farmers (Fig. 4A and SI Appendix, Table S4). Based on exchange of fry and selection of parental carp (SI Appendix, Surveys on the Ways of Parental Carp), three patterns of connection were evident among these three types of RF-farmers (Fig. 4B). Population viability analysis (PVA) (SI Appendix, Population Viability Analysis) showed that expected He of this indigenous carp can be maintained for a long time by a farmer who interconnected with other farmers, but cannot be maintained for long by an isolated farmer with a small number of parental carp (Fig. 4C). PVA further showed that large numbers of connected small farmer householders contributed to the maintenance of high He (Fig. 4D). PVA also showed that the connection intensity (indicated by frequency of exchange; see SI Appendix, Table S11) among these small farmer householders helps maintain the high He (Fig. 4E).

Population Performance and Yield of Color Types in a Field Experiment. Our field survey showed that about 65% of the RF-farmers cultured a mixture of PF-carp color types and most often cultured a mixture of two or three color types (SI Appendix, Fig. S5). In a field experiment, we compared the performance of PF-carp in plots that contained one of three color types or in plots that contained a mixture of all three color types.

In plots with only one color type (monocultures), growth rates significantly differed among the three color types (\( F = 10.282, P = 0.012 \)). In plots that contained the mixture, however, the growth rate did not significantly differ among the color types (\( F = 2.661, P = 0.149 \)).

Observation by video-recording technology showed that the frequency of total feeding activities did not significantly differ
among the three color types in mixture ($F = 0.777$, $P = 0.473$; Fig. 5B). The proportion of feeding activity represented by each of the three types of feeding activities, however, significantly differed among the three color types ($\chi^2 = 25.972$, $P = 0.0003$; Fig. 5B). The red type and the red–black type tended to spend more time than the black type foraging in the bottom mud (pie charts in Fig. 5B).

Stable isotope analysis (SIA) of $^{13}$C and $^{15}$N showed that the three color types had different values of $\delta^{13}$N and $\delta^{15}$C. The analysis of Stable Isotope Bayesian Ellipses in R (SIBER) also showed a separation of isotope niche, indicating that the three color types differed in food resources (Fig. 5C). Food source analysis further indicated that diet composition significantly differed among the red, red–black, and black types (SI Appendix, Table S5). The red type and the red–black type apparently preferred the food on the mud sources (snails, tubifex worms, and other small invertebrates), while the black type tended to use the three types of paddy field food sources more evenly (SI Appendix, Table S5).

At the same culture density, fish yield was significantly lower in plots with the black type than in plots with the mixture of the three types or with the red or red–black type (one-way ANOVA: $F = 13.203$, $P = 0.002$; Fig. 5D). On the other hand, fish yield was significantly higher for the mixture than for the average across plots with only one type (linear contrast: $t = -3.928$, $P = 0.004$; Fig. 5D).

**Discussion**

Our study describes how the indigenous common carp (PF-carp) is managed in the traditional rice–fish coculture system in southeastern China. The common carp (*C. carpio*) is an important cultured species in Eurasia (23). It has adapted to various environments across a broad range of environments and has been bred into numerous strains for commercial production (23, 24). Evolving in the rice–fish coculture system in southeastern China, the PF-carp has become a distinct local population that is well adapted to the paddy field environment, which is characterized by shallow water, a spatial grid of rice plants, and generalized food sources (Fig. 1) (21, 25). This PF-carp has high genetic diversity and diverse color phenotypes; the color types differ in their performance in the rice field (Figs. 1, 2, and 5). Although the PF-carp is genetically and phenotypically diverse, it exists as a continuous metapopulation with high gene flow among the small populations (village as a unit) in our study area (Fig. 2).

Researchers have studied how a large genetic diversity of crops and livestock can be maintained in other traditional agricultural systems (3, 8, 9). Research has shown that smallholders in traditional agricultural systems continually recognize, collect, and test new strains, resulting in the accumulation of diverse landraces or genotypes (4, 26). Farmer-to-farmer seed exchange channeled by marriage and kinship and by ethnolinguistic and ethnic grouping also contributes to the maintenance of genetic diversity (7, 9). In this study, we found that the locally adapted ways in which small farmer householders incubate and use PF-carp greatly affect the genetic diversity of this fish (Fig. 6).

At this GIAHS site, traditional RF-farmers have created a unique sharing system in which RF-farmers interdependently select parental carp and produce and exchange fry (Fig. 6). Unlike crop seeds, which can be stored for long periods and which can be transmitted long distances by a single farmer (27), the parental carp that are used to produce fry for rice–fish coculture must be continuously cultivated, and that continuous cultivation benefits from cooperation between many farmers. On the one hand, parental carp maintenance and fry production is too demanding for many single, smallholder farmers. On the other hand, a single smallholder with a small number of parental carp will risk
positive correlation between LCP (indicating farmer connectivity) and genetic distance (Fig. 3C). These results indicate that farmer activities can shape the genetic pattern, resulting in a continuous metapopulation of PF-carp in the study area (Fig. 2 C and D). Farming practices could also contribute to the diverse colors and genetic diversity of PF-carp (Fig. 6). Farming practices (e.g., the use of multiple varieties and of different cultivation methods), which are shaped by economics, culture, and, in some cases, religion, have been shown to help conserve the genetic diversity of traditional crops and livestock (10, 11, 30). In our case, RF-farmers tend to culture several color types of PF-carp in a single field (SI Appendix, Fig. S5). Because different color types have different feeding behaviors and use different food sources in the field (Fig. 5B and SI Appendix, Table S5), the mixed culture of color types results in a wide range of resource use (a wide isotope niche; Fig. 5C) and high fish yield (Fig. 5D). The benefits from the mixed culture of PF-carp, in turn, encourage RF-farmers to continuously practice mixed culture. Of the surveyed farmers, 64% practiced mixed culture in their fields (SI Appendix, Fig. S5). This further encourages A and B farmers to select and maintain diverse color types of PF-carp in this area.

The preference of local farmers in selecting traits (e.g., morphological traits, yield, and quality) could also affect the genetic diversity of crops and animals (4, 26). In our local farmer survey (SI Appendix, Farmer Survey for the Preference of Local People), the preference for color types of PF-carp as food differed substantially among the surveyed sites (SI Appendix, Table S6), although the various color types were found in all of the sampled sites (Fig. 1C). This preference could also help maintain the diverse color types of the PF-carp.

Our results suggest that the practices through which parental carp are selected and conserved, how fry are produced and exchanged, and how multiple carp with multiple phenotypes are cultured in the same rice field have helped the indigenous PF-carp to thrive in the traditional rice–fish coculture system. All of these practices are rooted in the cultures of local traditional RF-farmers. Worldwide, traditional farmers have developed diverse and locally adapted agricultural systems (3, 31). These indigenous agricultural systems have supported substantial genetic diversity and species diversity of crops (2, 3). Such genetic resources are important for sustaining agricultural production and for the breeding of new varieties (32, 33). Thus, the value of traditional agricultural systems should be recognized before they are replaced by modern agricultural systems. Just as “biodiversity hotspots” have been recognized as important for the conservation of wild species (34), traditional agricultural systems should be recognized as important for the conservation of agricultural genetic resources and of local wisdom throughout the world.

Materials and Methods

Ethics Statement. We obtained permits to conduct this study from local government administrative offices of the three counties (Qingtian, Yongjia, and Ruian in south Zhejiang Province, China) that we surveyed. During the survey and experiment, the local government administrative and agricultural extension officers were kept updated on our research activities. Farmers were informed of the purpose of the study, and were informed that the survey process only involved anonymous information. The farmers consented to participate in the study. Fish collection and experiments were carried out in accordance with the approved guidelines of Zhejiang University Experimental Animal Management Committee.

Farmer Survey. We conducted a survey on the activities of traditional RF-farmers in the site of the GIAHS rice–fish coculture system located in south Zhejiang Province (120°26′E–121°41′E, 27°25′N–28°57′N, Fig. 1C). The study area is described in SI Appendix, Study Site and Rice–Fish Coculture System and Fig. S1. A stratified sampling protocol was applied with three strata (county, town, and village). The three neighboring counties that were surveyed (Qingtian, Yongjia, and Ruian) have a long history of conducting rice–fish farming. Based on government statistics, 31 towns were chosen to
represent all types of rice–fish farming and all types of natural and social conditions in the three counties (SI Appendix, Fig. S1). Three to 19 villages per town (13 towns total) were surveyed for basic information, including methods of rice–fish farming (the use of a single color type or multiple color types of carp), carp yield, the preference of farmers for color types of carp, and the methods of maintaining parental carp and of producing fry. We also recorded the numbers of RF-farmers who hold large numbers of parental carp and produce fry for market (A farmers), numbers of RF-farmers who hold only small numbers of parental carp and produce fry for themselves and their neighbors (B farmers), and numbers of RF-farmers who do not hold parental carp in a village (C farmers) (SI Appendix, Table S4).

Selection of Carp Population for Sampling. The PF-carp in a village were considered a population in this study. Thirty-three villages (SI Appendix, Fig. S1 and Table S4), representing 33 populations, were randomly selected from the 176 surveyed villages described in Farmer Survey; these 33 populations were further sampled to investigate color phenotypes, morphological traits, and genetic diversity of PF-carp.

Among the 33 sampled villages, about 37% of them (12 villages) had one A farmers, i.e., farmers who maintain large numbers of parental carp. This proportion of villages with A farmers was higher than the proportion among 176 villages, which had 11% of villages with A farmers. Because a sampled village with large numbers of parental carp may have higher genetic diversity, we conducted an evaluation of whether this sampling would result in biased estimates of genetic diversity (SI Appendix, Evaluation of the Sampling PF-Carp Population and Fig. S6).

Carp Sampling and DNA Extraction. For molecular analysis, carp were collected from the 33 villages. In each village, more than 30 individual fish were randomly collected from a total of three to five farmer households. At the sampling site, a 0.3-cm-long segment of the tail fin of each carp was clipped, placed in 95% ethanol, and kept at 4 °C during transport to the laboratory. In the laboratory, the samples were kept at −20 °C until DNA was extracted. Genomic DNA was extracted from carp samples using a DNA extraction kit (E-Zap kit; Sangon) and following the manufacturer’s protocol. All DNA samples were stored at −80 °C for further phylogenetic and microsatellite analysis.

Mitochondrial Gene Sequence and Phylogenetic Analysis. Primers were designed based on the sequence of the mitochondrial D-loop gene of the common carp from the National Center for Biotechnology Information (NCBI) (GenBank accession no. NC_001606.1) (SI Appendix, Design of the Primers for the D-Loop Gene). All DNA samples of PF-carp were submitted to Sangon Biotech, which conducted PCR analysis using the designed primers and mitochondrial gene sequences.

The D-loop sequences of the PF-carp samples were then submitted to NCBI for alignment by using Blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi). D-loop sequences of five common carp strains (SI Appendix, Accession ID of the Five Common Carp Strains) that were highly similar to the sequence of PF-carp were selected from the Refseq database in NCBI. The mitochondrial gene sequence data of our PF-carp and the five common carp strains were aligned by using Multalin. A phylogenetic tree was constructed by using the maximum likelihood method. Confidence levels in the resulting relationship were assessed using the bootstrap procedure with 999 replications. All of these analyses were carried out in Mega 6.0 (35).

Survey of the Color Type and Morphology of PF-Carp. In October, when rice and fish were harvested, three to five rice fields in each of the 33 sampled villages (125 rice fields in total) were surveyed. The numbers of fish of each color type were recorded. At the same time, 267 carp individuals from the 125 rice fields were collected and transported to the laboratory for morphological trait measurement. All of these carp, which included all color types, had 2-y growth circles. Each individual was photographed with a digital camera (Canon EOS 60D), and the photographs were processed with tpsDig v.2.12 software (36) to measure the following traits: body length (Lb), head length (Lh), eye diameter (Dv), caudal peduncle length (Dpl), caudal peduncle length (Dps), dorsal fin ray number (Ndp), dorsal fin spine number (Nds), anal fin ray number (Nnf), and anal fin spine number (Nns). Eight indices (Lbf/Lb, Dvf/Lb, Dps/Lb, Lh/Lb, Ndp/Nbf, Nds/Nps, Nnf/Nps, and Nns/Nps) were used to describe the morphological traits.

To compare the morphological traits of PF-carp with those of five other strains of common carp cultivated in non-rice-field habitats, we obtained morphological data for these five common carp strains by conducting a literature review (SI Appendix, Extraction of Morphological Trait Data from the Literature). These five common carp strains were the same as the in the phylogenetic analysis. Using cluster analysis of the eight morphological indices (SI Appendix, Table S1), we evaluated the differences and distances between PF-carp and the five common carp strains.

Landmark-based geometric morphometrics was used to evaluate the morphological shapes of the mean three color types of the PF-carp (red, red-black, and black) (SI Appendix, Landmark-Based Geometric Morphometrics for PF-Carp and Fig. S7).

Microsatellite Genotyping. The microsatellite polymorphism of DNA samples of PF-carp was analyzed by using 20 pairs of microsatellite primers (SI Appendix, Table S7). Amplification of DNA was performed in 25-μL reactions containing 2.5 μL of 10× PCR Buffer (containing genetic structure, and individual assignment. The numbers of genetic groups (K value) were assessed [SI Appendix, Assessment of the Number of Genetic Groups (K Value)]. Geographical units (natural villages in the current study) were assigned to the clusters.

Evaluation of Genetic Diversity. Standard measures of genetic diversity were assessed for each population based on 20 loci. These measures included mean number of alleles per locus (Na), the effective number of alleles (Ne), observed (Ho) and expected (He) heterozygosity, and the inbreeding coefficient over loci (Fis). Genetic differentiation among populations was estimated from the fixation index (Fst) and analogs (G’st and Dst; ref. 37). These calculations were performed with GenALEX 6.502 (38).

The genetic diversity indices (He and Na) of PF-carp at the village level were compared with those of wild carp and cultured carp that live in non-rice-field habitats. He and Na values for these wild carp and cultured carp (SI Appendix, Table S8) were obtained from a literature review (SI Appendix, Genetic Diversity Indices of Other Common Carp). The validity of these He and Na data was also evaluated (SI Appendix, Genetic Diversity Indices of Other Common Carp, Figs. S8–S10, and Table S9).

The Kruskal–Wallis test was used to statistically compare He and Na among PF-carp, wild, and cultured carp. Bonferroni correction was used to control type-I error in multiple comparisons. A pairwise Wilcoxon test was also used to compare He and Na of PF-carp with wild carp or cultured carp (SI Appendix, Pairwise Test of He and Na). All of these statistical analyses were conducted in R (39).

Evaluation of Genetic Structure. AMOVAs were used to assess genetic variation of PF-carp within populations, among populations, and among regions. The significance of F statistics was tested by permutation with 1,000 randomizations.

Genetic distance was estimated based on Nei’s calculation (40). Based on genetic distance, PCoA was conducted to investigate the spatial pattern of genetic variability (41).

By using the nonspatial Bayesian cluster procedure in STRUCTURE 2.3.4 (42), we also investigated the identification of genetic structure and individual assignment. The numbers of genetic groups (K value) were assessed [SI Appendix, Assessment of the Number of Genetic Groups (K Value)]. Geographical units (natural villages in the current study) were assigned to the clusters.

Evaluation of Gene Flow. Gene flow among PF-carp in sampled villages (populations) was calculated by using the model of Wright (43). Nm = (1 − Fst × 4Fst), in which N is the effective population size, m is the migration rate, and Fst is a fixation index (indicating genetic differentiation among populations).

Estimation of Fry Flow Rate Within and Between Villages. For each of the 33 villages, we investigated the probabilities of carp fry being used within the village (Pin) or traded into other villages (Pout) (Pin + Pout = 1) through farmer interviews. At each village, we randomly interviewed 5 to 10 farmers (from separate households) who have been producing carp fry and of rice–fish coculture. During the interview, we asked three questions: (i) Do you sell or give fry to other farmers within or outside the village? (ii) Do you buy or receive fry from other farmers within and outside the village? (iii) Where do you obtain the fry that you use for rice–fish farming?

Based on the answers to these questions, we estimated the total numbers of fry used within the village (Pin) or traded between villages (Pout). We used Pout to indicate fry flow rate of a village.

Farmer Connectivity and Its Relationship to Genetic Distance. In agricultural systems, farmer connectivity greatly affects the dispersal of the dominant species (e.g., fish or livestock) and thereby their genetic structure. Farmer connectivity is affected by topography, market networks, and farmer activities.
Cost distance is a geographic information system (GIS) tool that allows the evaluation of “farmer connectivity” between locations by taking into account not only Euclidean distance but also farmer activity and other factors. Here, we used cost–distance modeling in ArcGIS 10.3.1 to evaluate farmer connectivity by estimating the LCP (SI Appendix, Estimation of the Least-Cost Path (LCP)). In this estimation, a low LCP value indicated high farmer connectivity.

Simple and partial Mantel tests were used to determine whether genetic distance could be explained by farmer connectivity (44, 45). In conducting partial Mantel tests, we first tested correlations between genetic distance and GD, and between genetic distance and LCP. We then assessed the correlation between the matrices of genetic distance and LCP while controlling for the effects of GD. Matrix of genetic distance was developed by using Nei’s method (40). Matrix of GD was developed by calculating Euclidian distance of geographic coordinates. P values were assessed by 10,000 pseudorandom permutations. These analyses were performed at the village level. Calculations were carried out using the package vegan from the R statistical program (39).

Maintenance of the PF-Carp and Population Viability Analysis. We conducted a survey of how farmers obtain fish fry and maintain parental carps for their fish–rice farming in the study area. In this survey, we focused on (i) numbers of adult carp (including parental carp) that usually are maintained by a farmer household; (ii) the number of connected farmer households that were involved in parental carp maintenance and fry production in a village; (iii) frequency of carp exchanges (parental carp exchange or selection, and fry transmission) among farmer households. We conducted this survey through farmer interviews (SI Appendix, Surveys on the Ways of Parental Carp).

We performed a population PVA to determine how the ways of PF-carp maintenance by farmer households affect genetic diversity in PF-carp populations. We used expected He to indicate the changes of genetic diversity in the PVA. Based on the farmer survey described in Farmer Survey, which found that farmer households were interconnected through parental carp selection and fry transmission (SI Appendix, Surveys on the Ways of Parental Carp and Table S11), we reasoned that numbers of connected farmer households, and connection intensity among farmer households, would affect He. In this PVA, we first compared the (i) partial Mantel of He under connected and not-connected farmer households (SI Appendix, Population Viability Analysis and Table S12). We then analyzed the effects of numbers of connected farmer households, and connection intensity among farmers (SI Appendix, Population Viability Analysis and Table S12). All PVA were conducted with Vortex 10.0.0.46 (46).

Field Experiment. We conducted a field experiment to assess the performance of the three major color types of PF-carp (red, red–black, and black) in single-color monoculture or in a three-color mixture in a rice field at the GIAS rice–fish system pilot site in Qingtian County (120°18′E, 27°59′N; see SI Appendix, Fig. S1). Rice yield in the rice–fish system at this pilot site averages about 6.15 ton·ha⁻¹ (19). The experiment, which lasted 5.3 mo (from May 1 to October 12, 2015), had a completely randomized block design with four treatments (three color types, six fry of each color (18 in total) were released into each plot. Blocks were independent of each other, i.e., they had different water inlets and outlets. Plots were separated by 50-cm-high concrete ridges. Four weeks after they germinated, rice seedlings (hybrid variety Zhong-Zhe-You no. 1) were transplanted into hills (one seedling per hill) within rows, with 30 cm between rows and 30 cm between hills in the same row. Fish fry, which were obtained from local farmers who maintained parental carp and produced fry, were re-leased 3 d after the rice seedlings were transplanted. For the monoculture of three color types, 18 fry (25 g each) were released into each plot. For the mixture of color types, six fry of each color (18 in total) were released into each plot. Plots were flush irrigated in 15 cm depth at transplanting. Then, the field water gradually was increased to 30 cm depth as fish grew up. A basal fertilizer (N:P:K fertilizer; 15:15:15, 150 kg·ha⁻¹) was broadcast in all plots before furrowing. No pesticide or top-dress fertilizer was used. During the whole experiment, no fish feed was applied.

At the rice-rieling stage, we used an above-water video system (SONY DCRSX21E) to monitor carp behavior in the plots with mixtures. A continuous recording (from 8:00 A.M. to 6:00 P.M.) was obtained for each mixture plot for 15 d (SI Appendix, Recording of PF-Carp Behavior in the Field). The frequency of carp activity per day in a 1-m² quadrat and the types of carp feeding behavior (foraging on the water surface, on the base of rice stems and leaves, and on the bottom mud) were assessed by viewing the recordings on a computer (SI Appendix, Recording of PF-Carp Behavior in the Field). Differences in the frequencies of feeding activities among the three color types in mixture were statistically assessed. The frequency data were square-root transformed for normality. A χ² test was conducted to compare the proportion of total feeding behavior represented by each of the three types of behavior among the three color types. All of the statistical analyses were completed in R (39).

We determined how the carp use food resources (e.g., phytoplankton, benthos, and algae) in the rice field by analyzing the stable isotopic composition (¹³C and ¹⁵N) of the living resources and of the carp (47). Living organisms that we assumed to be foraged by carp were collected every month during the rice-growing period (SI Appendix, Sampling of Fish Food Sources). The carp were also collected and analyzed for stable isotopic content (¹³C and ¹⁵N) before and after the experiment (SI Appendix, Sampling of PF-Carp for Stable Isotopic Analysis).

The dietary contributions of the potential food sources in the field were assessed by SIA and through dietary reconstruction in R with the package SIBER (49). To examine the diet composition of the three color types, we assigned the natural food sources in the rice field to three groups. Group I included foods that were mainly located on the water surface: duckweed, rice pollen, phytoplankton, etc. Group II included foods that were mainly located on the bases of rice stems and leaves: spirogyra and other algae. Group III included foods that were mainly located on the mud at the bottom of the field: snails, tubifex worms, etc. In the dietary reconstruction, the discrimination factors of ¹³C or ¹⁵N for carp were 2.73% and 1.71%, respectively (52). The Kruskal–Wallis test was conducted to compare the contribution (percent) of each food component to the diet among the three color types (39).

During the experiment, no fish mortality occurred. The carp growth rate was estimated by measuring the biomass of each individual carp before and after the experiment. Growth rate (percent) = (Wf – W0) × 100/W0, in which W0 was the average fry weight before the experiment, and Wf was the average fry weight after the experiment. Yield of carp was estimated by harvesting whole plots. The yield was expressed as tons of fresh carp per hectare. Data for fish growth rate and yield were subjected to a homogeneity test and were log-transformed if they did not meet the assumptions of normality and homogeneity of variance. ANOVAs were conducted to analyze the differences in carps growth rates and yields among the monocultures and mixture. Linear contrast was used to compare fish yield under the mixture of three color types with the average fish yield across plots with only one color type. The statistical analysis was completed in R (39).

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