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Research Article

Revealing Natural Hybridization Between *Hypophthalmichthys molitrix* and *Hypophthalmichthys nobilis* Within Their Native Range (Xiangjiang River of China) Through Morphological and Molecular Approaches

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Hypophthalmichthys molitrix and *Hypophthalmichthys nobilis* are economically important freshwater fish species in China. During resource surveys in the Xiangluzhou section of Xiangjiang River, Changsha, in 2020, fish suspected to be natural hybrids of *H. molitrix* and *H. nobilis* were identified. To confirm this, we employed morphological and molecular analyses of *H. molitrix*, *H. nobilis*, and their suspected hybrids. Morphometric results revealed that most countable and measurable traits of suspected hybrids ($n = 29$) were intermediate between those of *H. molitrix* ($n = 30$) and *H. nobilis* ($n = 26$). However, the traits were slightly biased toward those of *H. molitrix*, representing an intermediate, transitional form indicative of hybridization. Genome resequencing followed by principal component analysis and population structure analysis indicated that the genetic composition of the suspected hybrids ($n = 9$) was intermediate between *H. molitrix* ($n = 10$) and *H. nobilis* ($n = 9$), containing nuclear genetic material from both species. These results confirmed the suspected hybrids as true hybrids of *H. molitrix* and *H. nobilis*. COI gene barcoding results showed that the suspected hybrids ($n = 28$) formed a single clade in the haplotype network and shared haplotypes exclusively with *H. molitrix* ($n = 29$), indicating that *H. molitrix* was the maternal parent. Furthermore, the asymmetric hybridization pattern suggested potential influence of physicochemical factors, such as water temperature, water quality, and flow velocity, which affect the affinity between sperm and egg cells. We propose that the natural hybridization of *H. molitrix* and *H. nobilis* originates from genetic exchange. The influence of Xiangjiang River's unique aquatic ecological environment and hydraulic engineering projects, which have led to changes in flow regimes and the degradation of spawning grounds, have further altered the species' ecological reproductive habits and disrupted reproductive isolation and natural hybridization. This study provides direct empirical evidence of natural hybridization between *H. molitrix* and *H. nobilis* within their native ranges in China through morphological and molecular research methods, thereby complementing previous hybridization inferences based on genomic data. These findings offer a novel view on fish hybridization theory.

Keywords: asymmetric hybridization; genome resequencing; *H.* molitrix; *H. nobilis*; mitochondrial COI gene; natural hybrids

1. Introduction

Natural hybridization is a major driving force of biological evolution and plays a significant role in species diversification [1]. Hybridization among different fish species or genera is relatively common. Examples include hybridization between *Cyprinus carpio* and *Carassius auratus* [2], *Brachymystax lenok* and *Hucho taimen* [3], *Hemibarbus maculatus* and *Hemibarbus labeo* [4], *Schizothorax waltoni* and *S. o'connori* [5], and *Parasilurus asotus* and *Silurus* *soldatovi* [6]. Natural hybridization can act as a double-edged sword and is implicated in genetic introgression [7], genetic homogenization [8], population decline [9], and species extinction [10]. Conversely, it also contributes to an increased evolution in genetic diversity, enhanced environmental adaptability, emergence of novel phenotypic traits [11], and formation of new species [12].

Hypophthalmichthys molitrix and *Hypophthalmichthys nobilis*, belonging to the family Cyprinidae, subfamily Hypophthalmichthyinae, and genus *Hypophthalmichthys*, are economically important freshwater fish species in China. They are widely distributed in major river systems such as the Heilongjiang, Yangtze, and Pearl River basins [13]. As filter-fed fish, they play a crucial role in regulating aquatic ecosystems and are commonly used in polyculture systems. In 2023, the aquaculture production of *H. molitrix* and *H. nobilis* reached 3.86 and 3.35 million tons, respectively, accounting for 13.93% and 12.09% of the total freshwater fish production, second only to *Ctenopharyngodon idella* [14]. *H. molitrix* and *H. nobilis* share similarities in their life histories, reproductive behavior, and karyotypes [15]. Based on genomic data, genetic introgression from *H. nobilis* to *H. molitrix* has been reported in China [16]. However, natural hybridization between the species has not been documented, and they exhibit reproductive isolation in natural waters. In the 1970s, the United States introduced several freshwater fish species from China, collectively referred to as "Asian carp," which have since become established in natural water bodies. Notably, natural hybridization between *H. molitrix* and *H. nobilis* has been documented within the Mississippi River basin [17–19], with hybridization rates of up to 22.5% [20] and predominantly involving *H. molitrix* as the maternal parent. Xiangjiang River, a major tributary of Yangtze River that flows into Dongting Lake, is rich in fishery resources. It is home to eight national-level aquatic germplasm reserves, including those for the "Four Major Chinese Carp," and serves as a critical spawning, feeding, and migratory corridor for aquatic organisms. It is one of the three major spawning grounds for the "Four Major Chinese Carp" in China [21], with significant economic, ecological, and scientific value. In 2020, researchers discovered a fish with intermediate morphological and coloration traits between *H. molitrix* and *H. nobilis* during long-term germplasm preservation efforts. The abdominal ridge length of that fish falls between that of *H. molitrix* and *H. nobilis*, which is distinctly different from the complete abdominal ridge of *H. molitrix* and the incomplete abdominal ridge of *H. nobilis*. It was initially speculated to be a natural hybrid of *H. molitrix* and *H. nobilis*. Since the specimens collected from Xiangjiang River were newly hatched postlarvae lacking horizontal swimming ability, and considering the absence of hybrid larva production facilities for *H. molitrix* and *H. nobilis* upstream or in the surrounding areas of the sampling site, we hypothesized based on the evidence above that the suspected hybrids are the result of natural hybridization rather than artificial hybridization.

Hybrid offspring typically inherits genetic material from both parents [22], and their morphological traits often fall between those of the parental species [23, 24], making morphology a useful criterion for identifying natural hybrids. Genome resequencing is a high-throughput sequencing-based method [25] that involves resequencing the genomes of individuals from species with known reference genomes to identify genetic variations, such as single nucleotide polymorphisms (SNPs) [26]. This technique serves as an effective marker for identifying natural hybrids. Additionally, the mitochondrial *COI* gene, which follows maternal inheritance in fish [27], provides critical information for determining the maternal lineage. Molecular techniques are powerful tools for studying the genetic evolution of natural hybrids and species identification [28, 29]. The aim of this study was to compare the morphological characteristics of *H. molitrix*, *H. nobilis*, and their suspected natural hybrids in Xiangjiang River. By integrating genome resequencing and COI gene barcoding, we sought to analyze the genetic relationships of the suspected hybrids and confirm their identity from both morphological and genetic perspectives. We further aimed at elucidating the underlying causes of natural hybridization. Our findings enrich theory on species formation and provide a theoretical foundation for the conservation and utilization of *H. molitrix* and *H. nobilis* germplasm resources in Xiangjiang River.

2. Materials and Methods

2.1. Sample Collection and Preservation. From May 1 to June 30, 2020, an early resource survey of fish was conducted on the left bank of Xiangjiang River downstream the Xiangluzhou section in Changsha (112°53′5″E, 28°20′17″N; Figure 1). Suspected hybrid fry of *H. molitrix* and *H. nobilis* was collected and cultivated until adulthood in a dedicated pond at Hunan Fisheries Research Institute and Aquatic Products Seed Stock Station. Random samples of *H. molitrix* (HMCJ), *H. nobilis* (HNCJ), and their suspected natural hybrids (Hybs) were selected (Table 1) for morphological analysis, genome resequencing, and COI molecular experiments. Dorsal fin clips were taken, fixed in anhydrous ethanol, and stored at −20°C for further use.

2.2. Morphological Analysis. Following the Inland Fishery Natural Resources Survey Manual [30], Vernier calipers were used to accurately measure the following morphological traits in the three fish groups: total length (TL), body length (BL), abdominal keel length (AL), head length (HL), snout length (SL), eye diameter (ED), interorbital distance (ES), body depth (BD), caudal peduncle length (CL), and caudal peduncle depth (CD). Countable traits, including the number of dorsal fin rays (DRC), anal fin rays (ARC), lateral line scales (SNO), scales above the lateral line (SNA), and scales below the lateral line (SNB), were also recorded. Morphological data were analyzed using one-way analysis of variance in Excel 2017 and SPSS 19.0. Principal component analysis (PCA) and graphing were performed using Origin 2021.

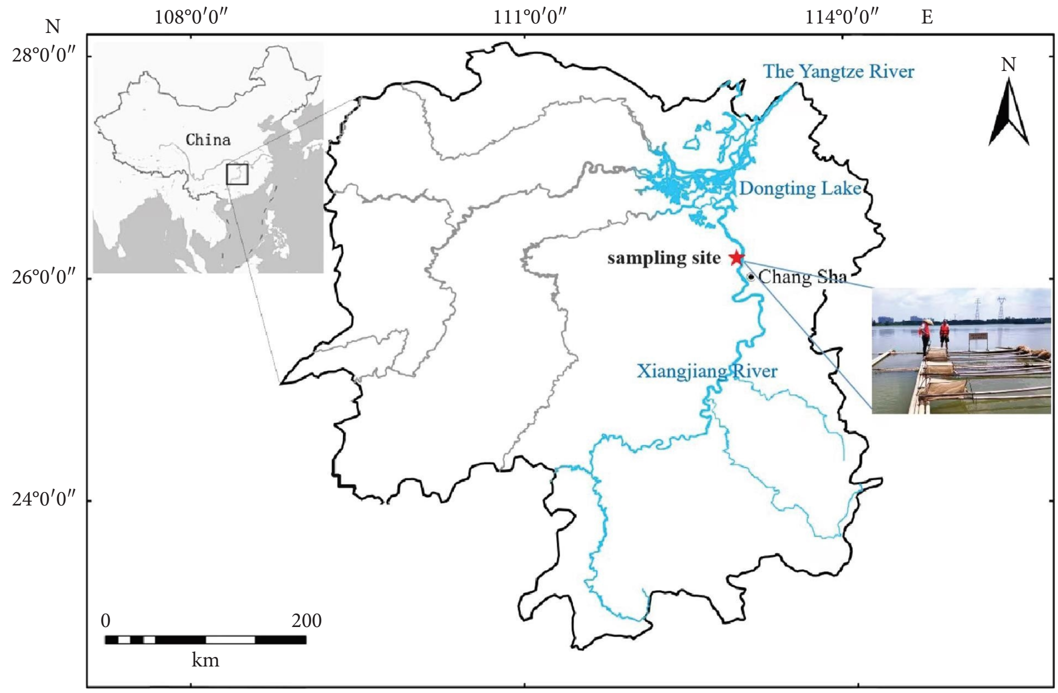

Figure 1: Location of the sampling site at the Xiangluzhou section of Xiangjiang River in Changsha City.

2.3. Genome Resequencing

2.3.1. Genomic DNA Extraction, Sequencing, and Library Construction. Genomic DNA was extracted from the dorsal fins of HMCJ, HNCJ, and Hyb using an animal tissue genomic DNA kit. DNA quality was assessed using 1% agarose gel electrophoresis, and DNA concentration and purity were measured using a Nanodrop D2000 spectrophotometer. The samples were then sent to Wuhan Benagen Technology Co., Ltd. for sequencing. Libraries were prepared using the Plus DNA Library Prep Kit for MGI V2 (#NDM627), following the manufacturer's protocol. Briefly, approximately 1 μg of genomic DNA was fragmented into 300–500 bp pieces using ultrasonication. The fragments were subjected to end repair, poly A tailing, adapter ligation, purification, and PCR amplification to complete library preparation. The qualified libraries were sequenced on the DNBSEQ-T7 platform using paired-end sequencing. Raw data were filtered to remove adapter sequences, duplicates, reads with an N content > 5%, and low-quality reads (where > 50% of bases had a quality score ≤ 5), resulting in clean reads.

2.3.2. Sequence Alignment and Evolutionary Analysis. Clean reads of HMCJ, HNCJ, and Hyb were aligned to the *H. molitrix* genome (GCA_037950675.1) using the Bowtie2 software [31], mapping sequences to their corresponding genomic positions. The sequencing depth and genome coverage were calculated. SNP detection was performed using GATK v4.1 [32] and Samtools v1.9 [33]. PCA was conducted using the Plink v1.9 software [34], and the results were visualized using the ggplot2 package in R. The nucleotide diversity (p) and genetic differentiation (Fst) for each population were calculated using Vcftools v0.1.16 [35]. Population genetic structure was analyzed using the Admixture v1.3 software [36], for which the optimal number of subpopulations (*K*-value) was determined based on the lowest cross-validation error rate (CV error). The results were visualized using the pophelper R package [37].

2.4. COI Gene Analysis

2.4.1. Genomic DNA Extraction and PCR Amplification. Genomic DNA was extracted from HMCJ, HNCJ, and Hyb using a commercial DNA extraction kit. The DNA concentration and purity were measured, and the quality was assessed. The *COI* gene was amplified using the universal fish primers reported by Ward et al. [38] (F1: 5′-TCAACCAAC CACAAAGACATTGGCAC-3′ and R1: 5′-TAGACTTCT GGGTGGCCAAAGAATCA-3′). The PCR reaction mixture (50-μL total volume) consisted of 5 μL of 10x buffer, 3 μL of 25 mmol/L $MgCl_2$, 1 μL of 10 mmol/L dNTPs, 2 μL each of 10 mmol/L forward and reverse primers, 2.0 U of Taq polymerase, and 1 μL of DNA template (100 ng/μL), with the remaining volume filled with double-distilled water. The PCR conditions were as follows: initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 30 s, and final extension at 72°C for 5 min. The PCR products were separated using agarose gel electrophoresis and the target bands were purified using a column-based DNA recovery kit. Purified PCR products were sent to Wuhan Tianyi Huayu Gene Technology Co., Ltd. for sequencing.

2.4.2. Data Analysis. The obtained DNA sequences were corrected for forward and reverse strands, and primer sequences were trimmed. Sequence alignment and analysis

Table 1: Sample Information of *H. molitrix*, *H. nobilis*, and their suspected hybrids from Xiangjiang River.

Species	Abbreviation	Sampling site	Sampling date	Sample size		
				Morphological analysis	Genome resequencing	*COI* gene
H. molitrix	HMCJ	Xiangjiang River	June 2022	30	10 (HMCJ01-10)	29 (HMCJ01-29)
H. nobilis	HNCJ	Xiangjiang River	June 2022	26	9 (HNCJ01-09)	26 (HNCJ01-26)
Suspected hybrid	Hyb	Xiangjiang River	June 2022	29	9 (Hyb01-09)	28 (Hyb01-28)

were performed using Clustal W [39]. The number of haplotypes (H) and segregating sites (S) were calculated using DnaSP 5.0 [40]. A haplotype network was constructed using Popart 1.7 [41]. Genetic distances within and between populations were calculated using MEGA11 [42], and a haplotype phylogenetic tree was constructed based on the Kimura-2-parameter model using the maximum likelihood method with bootstrap testing performed for 1000 replicates. Molecular variance analysis was performed using Arlequin 3.5 [43].

3. Results

3.1. Morphological Characteristics. As shown in Figure 2(a), for countable traits, the numbers of SNO, SNA, and SNB in Hyb fell between those in HMCJ and HNCJ. The number of ARC in Hyb was higher than that in HMCJ and HNCJ, whereas the number of DRC in Hyb was consistent with that in both HMCJ and HNCJ. Chi-square tests revealed significant differences ($p < 0.05$) between Hyb and both HMCJ and HNCJ in SNO and the number of ARC. For SNA and SNB, Hyb showed significant differences ($p < 0.05$) compared with HNCJ but no significant differences compared with HMCJ.

As shown in Figure 2(b), the BL/HL, HL/ED, HL/SL, HL/ES, BL/AL, and CL/CD ratios in Hyb fell between those in HMCJ and HNCJ. The BL/BD and BL/CL ratios in Hyb were higher than those in HMCJ and HNCJ, whereas the TL/BL ratio in Hyb was consistent with those in both HMCJ and HNCJ. Chi-square tests indicated significant differences ($p < 0.05$) between Hyb and HMCJ/HNCJ in the BL/HL, HL/ED, HL/SL, HL/ES, and BL/AL ratios. The BL/CL and CL/CD ratios differed between Hyb and HNCJ ($p < 0.05$) but not between Hyb and HMCJ. The BL/BD ratio between Hyb and HMCJ differed ($p < 0.05$) but not between Hyb and HNCJ.

As shown in Figure 3, PCA indicated that Hyb individuals were positioned between HMCJ and HNCJ, with closer proximity to HMCJ. Some Hyb individuals overlapped with HMCJ. PC1 was primarily influenced by AL and head-related traits such as HL and SL.

3.2. Genome Resequencing. PCA was performed on the SNP data from HMCJ, HNCJ, and Hyb. A PCA scatter plot (Figure 4(a)) was generated by assigning different colors to each group. The results showed that PC1 clearly separated the three groups into distinct clusters corresponding to HMCJ, HNCJ, and Hyb. The Hyb cluster was positioned between HMCJ and HNCJ, indicating that its nuclear genetic composition was intermediate between those of the two parental species, without bias. This suggests that Hyb inherited nuclear genetic material equally between HMCJ and HNCJ.

Population genetic analysis revealed that the CV error was minimized when $K = 2$, indicating that the population could be divided into two distinct subpopulations. The population structure plot (Figure 4(b)) demonstrated that Hyb exhibited two genetic backgrounds, containing genetic material derived from both HMCJ and HNCJ. This finding is consistent with the PCA results, confirming that Hyb represents a hybrid between HMCJ and HNCJ. The genetic differentiation plot (Figure 4(c)) showed a high genetic differentiation (Fst > 0.5) between the HMCJ and HNCJ populations, whereas the genetic differentiation indices between Hyb and HMCJ, as well as between Hyb and HNCJ (Fst < 0.5), were relatively low. This indicates a close genetic relationship between Hyb and the two parental populations (HMCJ and HNCJ). A nucleotide diversity plot (Figure 4(d)) revealed that overall, all three populations exhibited relatively low nucleotide diversity ($p < 0.02$), with Hyb showing slightly higher nucleotide diversity than both HMCJ and HNCJ.

3.3. COI Gene Barcoding. A total of 83 *COI* gene fragment sequences were obtained by PCR amplification. After alignment and trimming using Clustal W, a 696 bp effective fragment was retained. In HMCJ, 34 segregating sites were detected, accounting for approximately 4.88% of the total sequence length, and six haplotypes were identified. In HNCJ, two segregating sites were detected, accounting for approximately 0.29% of the total sequence length, and three haplotypes were identified. In Hyb, 12 segregating sites were detected, accounting for approximately 1.72% of the total sequence length, and two haplotypes were identified.

A haplotype network was constructed using the Popart 1.7 software (Figure 5). The results showed that the nine haplotypes formed two major branches corresponding to the HMCJ and HNCJ groups. The groups were highly differentiated, with 22 fixed mutational differences connecting species-specific haplotypes representing several single-nucleotide mutations. The haplotypes Hap-2, Hap-7, and Hap-8 clustered within the HNCJ branch, whereas the remaining haplotypes clustered within the HMCJ branch. In the suspected natural hybrid group, haplotype Hap-9 clustered within the HMCJ group and haplotype Hap-5 was shared between Hyb and HMCJ. This indicated that the suspected natural hybrids carried only the mitochondrial genes of HMCJ, confirming that their maternal parent was HMCJ.

4. Discussion

4.1. Analysis of Morphological Traits. Three main types of morphological variations in distant hybrid offspring of Cyprinidae fish have been reported: maternal-like [44, 45], paternal-like [46, 47], and intermediates between the two [48]. In this study, three out of the five countable Hyb traits were intermediate between HMCJ and HNCJ, one was consistent with both parents and one was higher than that in both parents. Among the nine measurable trait ratios of Hyb, six were intermediate between HMCJ and HNCJ, one was consistent with both parents, and two were higher than those in both parents. These results indicated that the most countable and measurable traits of Hyb were intermediate between the two parental species, consistent with the intermediate type. Some few traits exceeded or matched those

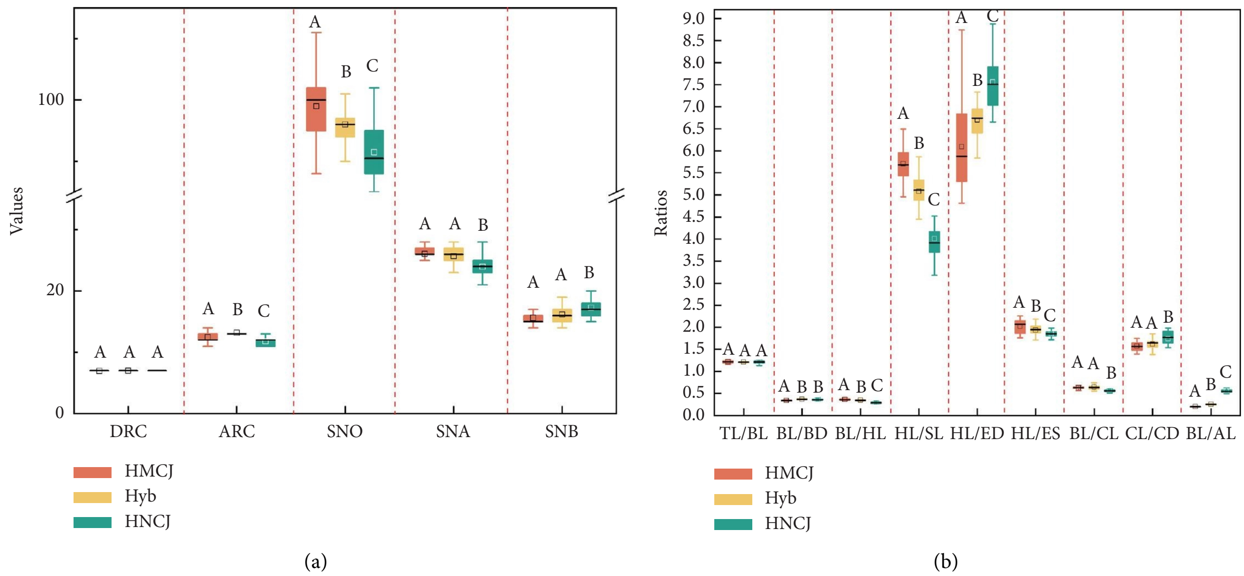

Figure 2: Comparison of major countable and measurable traits among HMCJ, HNCJ, and Hyb. (a) Boxplot of countable traits and (b) Boxplot of measurable traits.

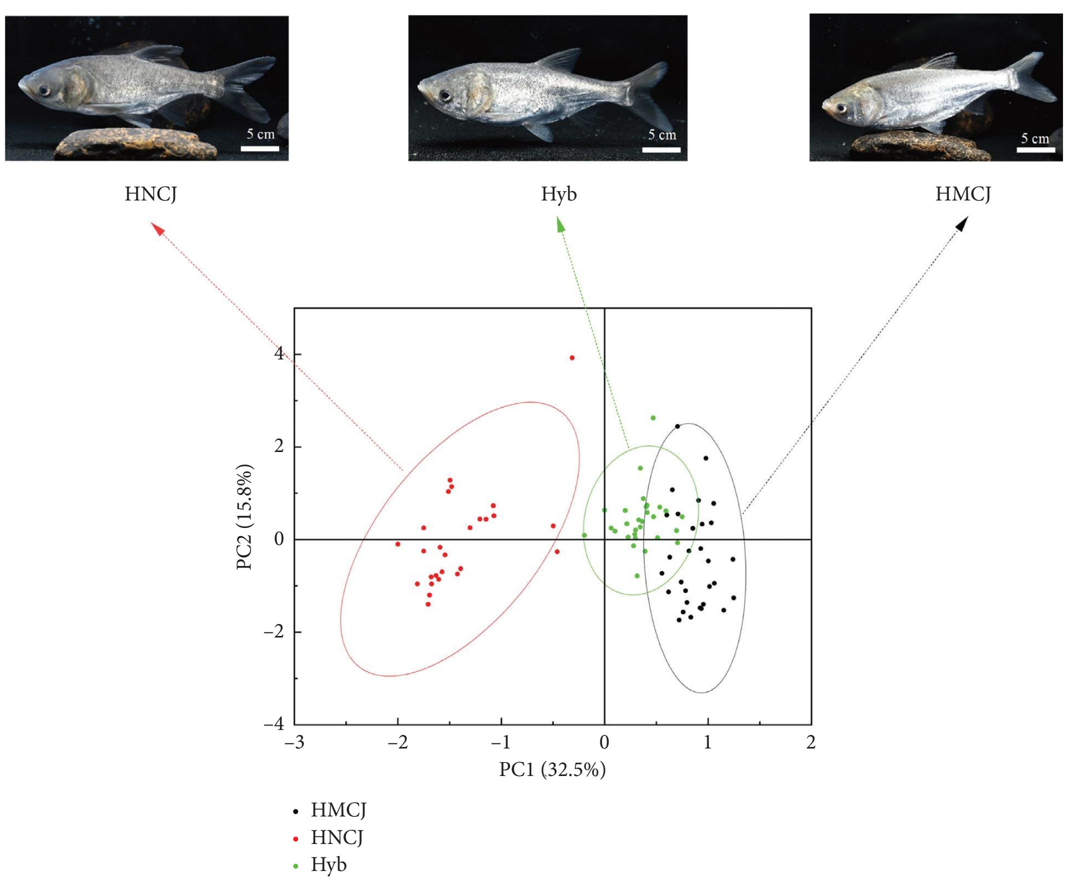

Figure 3: PCA of the morphology of HMCJ, HNCJ, and Hyb. Bar = 5 cm.

of the parents, which is consistent with the morphological characteristics of hybrid fish [49, 50]. PCA further validated these findings, showing that Hyb was more closely related to HMCJ. This result is consistent with the findings of Liu et al. [51], who analyzed the morphological differences between hybrid offspring of *H. molitrix* (♀) × *Hypophthalmichthys nobilis* (♂) and their parents. The morphological traits of fish represent the most direct external manifestations of the

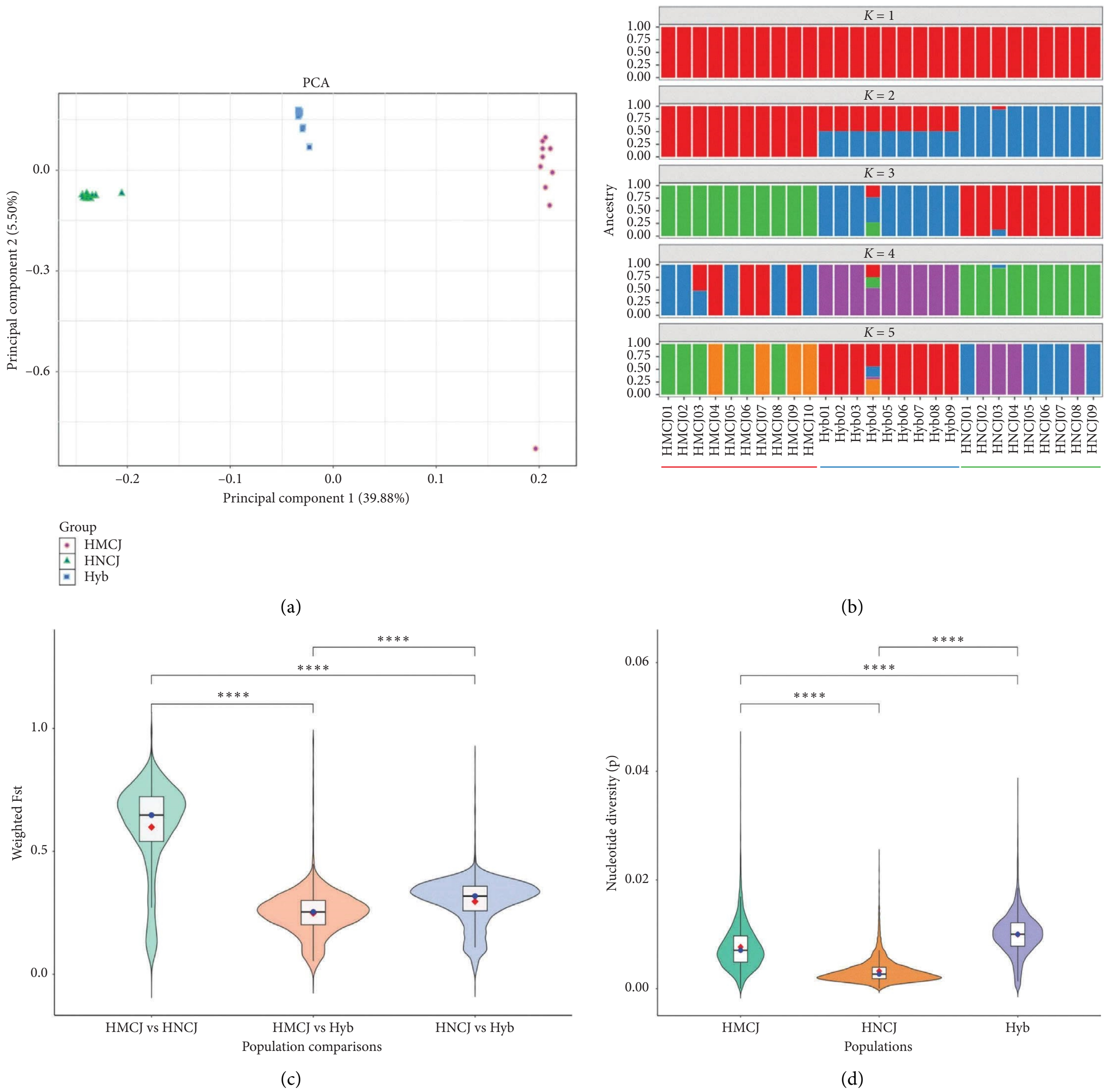

Figure 4: Population genetic analysis of HMCJ, HNCJ, and Hyb based on genome resequencing. (a) PCA plot of each individual. (b) Population structure ($K = 2$) of all individuals. (c) Pairwise genetic differentiation (Fst) between populations. (d) Nucleotide diversity index (p) for each population. Note: *indicates a significant difference.

genetic characteristics of a species. Although these traits can be influenced by habitat [52], they are predominantly determined by genetic traits [53]. Hyb inherited genetic material from both HMCJ and HNCJ, exhibiting intermediate morphological characteristics. This demonstrates the hybrid nature of Hyb, classifying it as an intermediate transitional type distinct from the specific variations within pure HMCJ or HNCJ individuals. The slight morphological bias toward HMCJ may be related to the inheritance of HMCJ mitochondrial genes by fertilized eggs during development [54]. In addition, a few traits exhibited transgressive variation (hybrid offspring outperform their parents in some traits) or stability (matching both parents), reflecting the stability of trait inheritance. In the present study, PCA identified several highly discriminative morphological traits, such as BL/VPL and BL/HL, which are useful for identifying and managing Hyb. Compared with the method relying on gill raker deformity for identification used by Lamer et al. [20], our PCA-based approach is scientifically robust, intuitive, reliable, and consequently more beneficial for germplasm resource conservation.

4.2. Genome Resequencing Analysis. Genome resequencing technology offers advantages such as high throughput and resolution, making it widely applicable for studying the genetic structure of populations. Analyses using this technology revealed that weedy rice (*Oryza sativa f. spontanea*

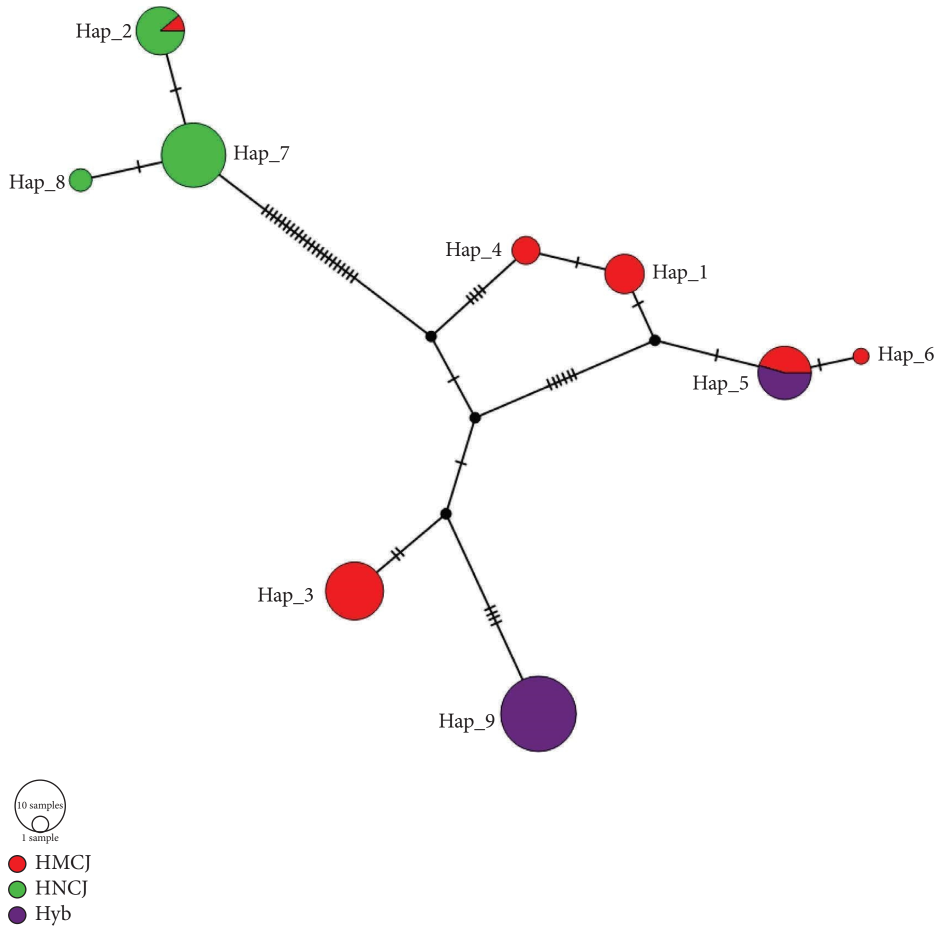

Figure 5: Haplotype network structure of the *COI* Gene. Note: Each circle represents a haplotype, with the size of the circle proportional to the number of samples included in that haplotype. The lines connecting the circles represent a single nucleotide change. Small black dots represent inferred haplotypes. Different colors represent species from different groups.

Rosh.) occupies an intermediate phylogenetic position and exhibits principal component values between japonica and indica rice varieties, supporting its hypothesized origin in indica–japonica hybridization [55]. Similarly, Chen et al. [56] used whole-genome resequencing to reveal evidence of hybridization between European and Indian cattle types. He et al. [57] also used genome resequencing to identify hybrid individuals within Black Angus cattle.

In the present study, PCA based on genome resequencing data effectively distinguished the three groups, clearly separating them into three distinct lineages. This indicates that Hyb is genetically distinct from HMCJ and HNCJ and should not be considered as an individual mutation in either species. The genetic composition of Hyb was intermediate between those of HMCJ and HNCJ, suggesting that Hyb is a transitional form between the two species. Moreover, population structure analysis revealed that Hyb possesses genetic material derived from both HMCJ and HNCJ, confirming that Hyb represents a hybrid between HMCJ and HNCJ. We further observed that nuclear genetic material from both parental sources contributed equally to the offspring during the natural hybridization process between these two species.

4.3. COI Gene Barcoding Analysis. The *COI* gene in fish is inherited maternally, making it a crucial marker for identifying maternal lineages in hybrid offspring [58, 59]. In the present study, the haplotype network constructed using the *COI* gene showed that Hyb clustered exclusively with HMCJ and shared haplotypes with this species. This confirmed that Hyb carries only the *COI* gene of HMCJ, indicating that the maternal parent is HMCJ. Similar findings have been reported previously by Yaakub et al. [60], who used the *COI* gene to confirm that *Halichoeres garnoti* acts as a maternal parent in natural hybridization with *H. bivittatus*. Yaakub et al. [61] used *COI* and other molecular markers to demonstrate natural hybridization between *Thalassoma quinquevittatum* and *T. jansenii*, with *T. quinquevittatum* as the maternal contributor. Marie et al. [62] used *COI* to support the occurrence of natural hybridization between female *Acanthurus nigricans* and male *A. leucosternon*.

Based on *COI* barcoding, only hybrids with HMCJ as the maternal parent and HNCJ as the paternal parent were identified in Xiangjiang River. This suggests the existence of an asymmetric hybridization pattern or insufficient sampling for the detection of reciprocal hybrids. Lamer et al. [20] found that nearly 88% of the natural hybrids between

H. molitrix and *H. nobilis* in the Mississippi River had *H. molitrix* as their maternal parent, which is consistent with our findings. Asymmetric hybridization may result from prezygotic isolation barriers [63], postzygotic effects [64], or a combination of both [65]. Avise and Saunders [66] suggested that asymmetric hybridization could arise from differences in the relative abundance of parental species, with rarer species being more likely to act as maternal parents. Vanhaecke et al. [67] proposed that the smaller body size of *Aplochiton zebra* may lead to sneaking behavior during reproduction, resulting in asymmetric hybridization with *A. taeniatus*. In Xiangjiang River, surveys have shown that *H. molitrix* is more abundant than *H. nobilis* in fishery catches [68], and the importance of *H. molitrix* larvae significantly exceeded that of *H. nobilis* [69]. Additionally, mature individuals of both species are similar in size [70]. Therefore, the asymmetric hybridization observed in this study may have also been influenced by other factors. Artificial hybridization experiments conducted on *H. molitrix* and *H. nobilis* revealed that the direct cross (*H. molitrix*♀ × *H. nobilis*♂) exhibited high fertilization, hatching, and survival rates during the summer fingerling stage. In contrast, the reciprocal cross (*H. nobilis*♀ × *H. molitrix*♂) achieved a fertilization rate of only 40%–50%, and the offspring displayed a significantly higher incidence of morphological abnormalities [71]. Fluctuations in water temperature, quality, and flow rate in natural water bodies such as Xiangjiang River may further affect gamete affinity, particularly during distant hybridization between *H. molitrix* and *H. nobilis*. This could reduce the fertilization and hatching rates of natural hybrids, rendering the survival and detection of reciprocal hybrids unlikely. Consequently, most of the natural hybrids collected in this study had *H. molitrix* as the maternal parent and *H. nobilis* as the paternal parent.

4.4. Investigation Into the Causes of Natural Hybridization. Natural hybridization in fish often occurs under specific conditions. For example, *H. molitrix* and *H. nobilis* introduced into foreign habitats show overlapping spawning periods and multiple spawning events within a year, thereby increasing the likelihood of natural hybridization [17, 20]. A shortage of male *H. nobilis* at the end of the breeding season may also contribute to hybridization between *H. molitrix* and *H. nobilis* [17]. In the unique habitat of Yarlung Zangbo River, spatially limited and overlapping spawning grounds can lead to natural hybridization between *S. waltoni* and *S. o'connori* [5]. Additionally, climate change and hydraulic engineering projects can significantly alter hydrological characteristics, disrupt reproductive isolation, increase contact frequency, and potentially induce natural hybridization [72]. In addition, anthropogenic stressors, such as environmental degradation or the introduction of non-native species, can disrupt ecological balance and promote hybridization [67]. For instance, increased turbidity in Lake Victoria disrupted visual-based mate selection and reproductive isolation among cichlids [73]. The release of farmed fish in the Iberian Peninsula may have facilitated hybridization among salmonids [74]. Finally, hybridization can occur when one species expands into the range of another without reproductive isolation [75].

In this study, naturally occurring hybrids of *H. molitrix* and *H. nobilis* from Xiangjiang River were identified. This phenomenon may be related to the distinctive aquatic ecological environment of Xiangjiang River, which influences the ecological reproductive behavior of these fish species. Genomic analysis revealed gene introgression from *H. nobilis* to *H. molitrix*, providing molecular evidence of natural hybridization between these species [16]. Moreover, natural hybridization between *H. molitrix* and *H. nobilis* has frequently been reported, confirming that their reproductive isolation may break down under specific environmental conditions [17, 20].

Since the 1990s, a cascade of hydropower projects has been constructed along the main stream of Xiangjiang River, with eight navigation-power junctions currently in operation and additional projects being planned. This intensive hydraulic engineering project fundamentally transformed the river's ecological conditions, converting a fast-flowing ecosystem into a chain of interconnected reservoirs. Consequently, habitat fragmentation has directly blocked the spawning migration routes of *H. molitrix* and *H. nobilis*, while disrupting gene flow between upstream and downstream populations across dam barriers [76]. Furthermore, these projects have altered the natural hydrological regimes of the river [77]. The substantial thermal buffering capacity of reservoirs has flattened the thermal regimes downstream of dams [78], thereby delaying the spawning activities of the major Chinese carps and disrupting their growth patterns [76]. Such alterations may induce phenological shifts in fish biological traits [79]. Concurrently, optimal spawning flow velocities and spawning-triggering flow thresholds for *H. molitrix* and *H. nobilis* have been reduced [80], and diminished flood peaks and attenuated rising rates during high-flow periods directly impact spawning timing. Studies on the reproductive biology of *H. molitrix* and *H. nobilis* in the Yichang section of Yangtze River have revealed that their peak spawning periods overlap, occurring from June to July [76, 81]. Moreover, hydraulic engineering projects have caused fragmentation and shrinkage of spawning grounds for the "Four Major Chinese Carps," accompanied by sharp declines in fry abundance [82, 83]. Statistical analyses revealed a 67% reduction in spawning ground length for drift-egg-spawning fish along the Xiangjiang mainstream [77], with intensified spatial concentrations leading to overlapping spawning areas between *H. molitrix* and *H. nobilis*. Notably, *H. nobilis* spawning habitats suffered the most severe degradation, constituting only 2% of the major carp fry collected. From 1998 to 2003, *H. nobilis* fry was completely absent for three consecutive years, with persistently low recruitment thereafter [68], which indicates the most critical germplasm resource decline among the four species, resulting in an imbalance in the abundance of *H. molitrix* and *H. nobilis*. Therefore, we reveal in this study, based on the genetic compatibility facilitating gene exchange between *H. molitrix* and *H. nobilis* in conjunction with the unique hydrological environment and hydraulic engineering projects in Xiangjiang River, that the ecological and reproductive behaviors of these two species have been altered. These changes include a significant overlap in spawning periods, shared spawning habitats, and an imbalance in population ratios, all of which contribute to the natural hybridization between *H. molitrix* and *H. nobilis* [84].

4.5. Ecological and Evolutionary Implications for Natural Hybridization. According to previous productive experiments [71], the F_1 generation artificially hybridized from *H. molitrix* (♀) × *H. nobilis* (♂) exhibited good survival rates and rapid growth, reaching sexual maturity within 4–5 years. Their reproductive systems developed normally, and they primarily fed on plankton, potentially leading to trophic competition with their parent species. Frequent natural hybridization of *H. molitrix* and *H. nobilis* in Xiangjiang River could threaten the genetic diversity of the parent species. This presents a critically important issue worthy of in-depth investigation. Although there is no direct evidence that frequent hybridization compromises the genetic integrity of the parent species, the current decline in population size and the potential negative impacts of hybridization cannot be overlooked. Furthermore, numerous cases exist where frequent hybridization led to species extinction and reduced biodiversity, such as in *Micropterus coosae* [85], *Pseudorasbora pumila* [86], and *Etheostoma osburni* [87]. Additionally, mitochondrial DNA studies suggest that populations introduced from Yangtze River threaten native stocks of *H. molitrix* and *H. nobilis* in the Pearl River [88]. This could lead to the replacement of one lineage with another through introgression and hybridization, negatively affecting the genetic diversity of native populations in the Pearl River. Hence, it is imperative to enhance genetic conservation measures for fish germplasm resources in Xiangjiang River, monitor trends in introgressive hybridization among species, and promptly develop effective strategies for future species conservation efforts.

5. Conclusions

This study provides direct evidence of natural hybridization between *H. molitrix* and *H. nobilis* within their native Xiangjiang River ecosystem in China. Morphological and genetic analyses revealed hybridization events predominantly with *H. molitrix* as the maternal parent. Hybridization likely stems from disrupted reproductive isolation driven by the river's unique hydrological conditions and anthropogenic impacts, notably hydraulic engineering projects, which have altered the aquatic environment and further affected the ecological habits of fish and their relative abundances. Although the current scale and ecological consequences for germplasm integrity and ecosystem stability remain uncertain, these findings require urgent implementation of conservation strategies, such as coordinated ecological operations of hydropower complexes to ensure spawning migration, prioritizing habitat restoration for declining *H. nobilis* populations, and targeted stock enhancement using native germplasm.

Data Availability Statement

The authors confirm that the data supporting the findings of this study are included within the article.

Ethics Statement

This study was approved by the Experimental Animal Management and Use Ethics Committee of Hunan Fisheries Research Institute and Aquatic Products Seed Stock Station. All procedures were conducted in strict compliance with the ethical guidelines of Hunan Fisheries Research Institute and Aquatic Products Seed Stock Station, following the regulations established by its Ethics Committee (procedure approval 13 January 2022, approval code HNFIAPS20220113).

Disclosure

A preprint has previously been published [84]. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

Author Contributions

Zhiliang Zuo, Lu Tian, and Feng Gao: methodology; Zhiliang Zuo and Lu Tian: software and writing–original draft preparation; Zhiliang Zuo: validation, formal analysis, writing–review and editing, visualization, and supervision; Qifan Wu: investigation; Zhiliang Zuo, Cheng Li, Qifan Wu, Jin Xiang, and Hewei Xiao: resources; Zhitao Peng and Feng Gao: data curation; Lu Tian: project administration; Feng Gao: funding acquisition.

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