RESEARCH ARTICLE

Introgressive hybridization between two close species *Siniperca chuatsi* and *Siniperca kneri* (Percomorpharia: Sinipercidae) in the Middle Reaches of the Yangtze River

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Abstract – *Siniperca chuatsi* and *Siniperca kneri* are two economically important freshwater fishes endemic to East Asia. Recently, some *Siniperca* specimens collected from Lake Poyang and Lake Dongting in the middle reaches of the Yangtze River couldn't be clearly identified as they showed intermediate morphological characteristics between *S. chuatsi* and *S. kneri,* and some inter-species hybrids were detected by microsatellite loci. To further verify genetic composition of these intermediate individuals, and determine the prevalence and degree of introgression between the two *Siniperca* species, a large set of high-quality, independent, diagnostic genetic markers were necessarily required. Based on enrichment and sequencing of target genes in sinipercid fishes, 463 loci ($F_{ST} = 1$) between *S. chuatsi* and *S. kneri* were selected and verified for species diagnosis. A total of 349 loci with 458 diagnostic SNPs were identified for discriminating *S. chuatsi* and *S. kneri*. From those markers, 224 diagnostic SNPs (only one SNP per locus) were selected to identify and categorize 48 specimens with intermediate morphological characters. The results showed that there were 8 specimens identified as hybrids, 8 specimens as *S. chuatsi* and 32 specimens as *S. kneri*. NEWHYBRIDS analysis showed that the hybrid offsprings were composed of the first-generation hybrid (2 individuals), first-generation backcross (1 individual), second-generation backcross (1 individual) and fourth-generation backcross (4 individuals), and the backcrossing could happen to both *S. chuatsi* and *S. kneri*. These hybrids could occur naturally, or escaped from farmed fish, due to extensive artificial breeding practice in these regions. However, the origin of the introgressive hybridization can't be easily traced. Therefore, some measures for protecting genetic resource of *Siniperca* speies in the Yangtze River should be enforced, such as assessing genetic background of the cultured stocks, reducing the escapement from farmed fish, and monitoring the trend of introgressive hybridization between *Siniperca* species in the future.

Keywords: *S. chuatsi* / *S. kneri* / introgressive hybridization / SNP / target gene sequencing

1 Introduction

Both mandarin fish (*Sinipeca chuatsi*) and big eye mandarin fish (*Siniperca kneri*) belong to Sinipercidae (Percomorpharia), which are unique freshwater fishes in East Asia with important economic and ecological values. *S. chuatsi* is mainly distributed to the northern area of the Minjiang River, while *S. kneri* is distributed in the river systems south of the Huaihe River, with their distribution overlapped in the river systems in central China. In morphology, *S. chuatsi* is highly similar to *S. kneri*, with compressed body, humped back, large oral fission, protruding lower jaw, densely serrated teeth in buccopharyngeal, strong dorsal fins, round and small scales. There only a few morphological difference in these two species: *S. chuatsi*, the maxillary bone extending to or over the posterior edge of the eye, small eye diameter, high humped back; *S. kneri*, the maxilla not reaching to the posterior edge of the eye, large eye diameter, and low humped back (Zhou et al., 1988) (Fig. 1).

As for other aspects, *S. chuatsi* and *S. kneri* are two close species. A comparative study on the skeletal characteristics of seven sinipercinae fishes showed that there was no significant difference between *S. chuatsi* and *S. kneri* (Kong, 1993). Both

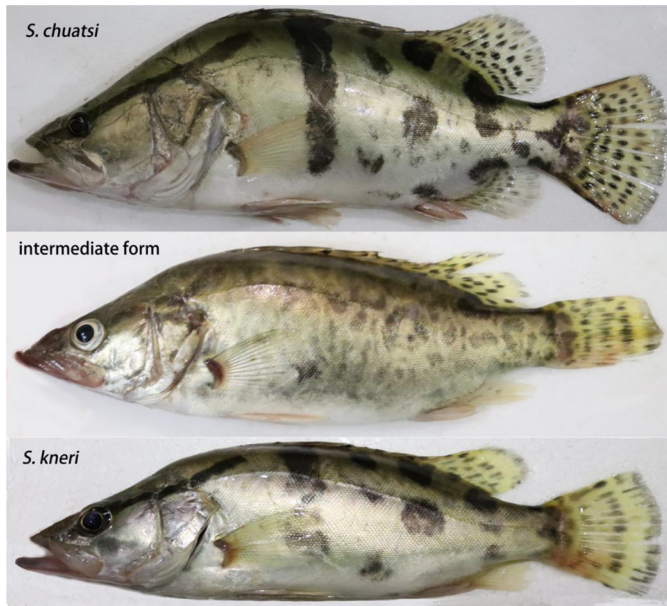*Corresponding author: jlzhao@shou.edu.cn

Fig. 1. Morphology of *S.chuatsi, S.kneri* and intermediate form (Zhu et al., 2020).

S. chuatsi and *S. kneri* are diploid (2n = 48) and have the same karyotypes (Yu, 1989). The size and complete mitochondrial DNA sequences of *S. chuatsi* and *S. kneri* were highly similar (Yin and Yang, 1988; Chen et al., 2012) and their genetic distance was very small (Zhao et al., 2006a, b; Wu et al., 2014; Zhao et al., 2015). The phylogenetic relationship of the sinipercids constructed using nuclear gene sequences also supported that *S. chuatsi* and *S. kneri* were sister species (Li et al., 2010; Song et al., 2017).

Lake Poyang and Lake Dongting are two largest lakes in the middle reaches of Yangtze River, which are the important breeding and nursing habitat for mandarin fishes. With increasing samplings of mandarin fishes from wild populations, we found that some specimens collected from these lakes had morphological characteristics intermediate between *S. chuatsi* and *S. kneri*, which brought difficulty in their classification. Yu (2011) firstly recognized these fishes as the "transitional species", since no significant genetic differentiation was detected between the "transitional type" and *S. chuatsi*, *S. kneri* at mitochondria DNA sequence level. Yang (2014) detected four hybrids specimens in *S. kneri* samples collected from the middle reaches of the Yangtze River (Lushui Reservoir, Ruanjiang River, Xiangjiang River, Ganjiang River, and Lake Poyang). Song et al. (2017) found there was some gene exchange in some mandarin fish populations of the Yangtze River. In our previous works, the morphometric analysis failed to identify intermediates specimens from Lake Poyang and Lake Dongting (Zhu et al., unpublished); however, there were some first-generation backcross recognized using five microsatellite markers (Zhu et al., 2020). Due to limited number of microsatellite markers and low site specificity, we were not able to fully resolve the classification of these intermediate specimens and understand the phenomenon of the introgressive hybridization between *S. chuatsi* and *S. kneri*. Moreover, their crossing types are not clear and informative for management of the genetic

resources of mandarin fishes. Therefore, a large set of high-quality, evolutionarily conserved, diagnostic genetic markers are required to further identification of these intermediate morphological individuals genetically, and to determine the prevalence and degree of introgression between the two *Siniperca* species.

SNPs are abundant throughout the genome, bi-allelic, evolving according to a simple mutational model (Vignal et al., 2002) with predictable rates of substitution and are highly conserved over the geographical range of an organism, thus ideal markers for hybrid analysis (Hohenlohe et al., 2011; Twyford and Ennos, 2012; Lamer et al., 2015). In our previous study, Song et al. (2017) applied the cross-species target gene enrichment method (Li et al., 2013) and obtained 16 943 single-copy nuclear gene sequences and thousands of single nucleotide polymorphisms (SNPs) loci in sinipercid fishes. The results showed that *S. chuatsi* and *S. kneri* were two valid species independent form each other. On this basis, this study screened SNP loci with a differentiation index, $F_{ST} = 1$, between *S. chuatsi* and *S. kneri*, and tested these SNPs in large samples to develop molecular diagnostic markers for *S. chuatsi* and *S. kneri*. These markers were used to carry out molecular identification of the intermediate forms from the lakes in the middle reaches of the Yangtze River, to explore the types of the hybrids and the direction of introgressive hybridization, which would provide scientific basis for monitoring and management of mandarin fish resources in the Yangtze River.

2 Materials and methods

2.1 Sampling

A total of 97 samples were collected from lakes in the middle reaches of the Yangtze River from March to September 2018, of which 72 samples were collected from Lake Poyang (Kangshan Town, Yugan County, Jiangxi Provine: 28° 52′ 56.87″N, 116° 25′ 53.54″E); 25 samples from Lake Dongting (Yueyang City, Hunan Province: 29° 23′ 49.69″N, 113° 05′ 46.07″E). In addition, six *S. kneri* samples were collected from the Pearl River (Sanfang Town, Huizhou City, Guangdong Province: 25° 15′ 0.21″N, 108° 50′2 8.03″E), and 8 *S. chuatsi* samples from the Amur River (Fuyuan City, Heilongjiang Province: 48° 21′ 44.60″N, 134° 18′ 0.15″E) (Tab. 1). All *S. chuatsi* and *S. kneri* specimens were identified based on the traditional morphological classification characters, and further verified with five microsatellite markers (Zhu et al., 2020). There were a total of 48 intermediate specimens unidentified. All animal handling procedures were approved by the Animal Care and Use Committee of Shanghai Ocean University and followed the guidelines of animal experiments on Shanghai Ocean University (SHOU-DW-2017-013). All fish were euthanized with 0.02% MS-222, then they were labeled and fixed with 95% ethanol.

2.2 DNA extraction

Genomic DNA was extracted from white muscle tissues using the TIANamp Marine Animals DNA Kit. Extracted DNA was dissolved in 120 μL ddH₂O. Concentration and quality of DNA extracts were measured using a Nanodrop 2000 spectrophotometer ($1.8 < OD_{260}/OD_{280} < 2.0$). DNA was then diluted to 20 ng/μL before stored at −20 °C.

Table 1. Sampling information of *S. chuatsi*, *S. kneri* and the intermediate form.

Collection location	Collection date	Number of samples		
		S. chuatsi	*S. kneri*	Intermediate form
Lake Dongting, Yangtze River	2018.9	7	8	10
Lake Poyang, Yangtze River	2018.3	8	8	9
	2018.9	8	10	29
Amur River	2018.2	8		
Pearl River	2018.7		6	
Total		31	32	48

Table 2. Assignment criteria for *S. chuatsi* (SC), *S. kneri* (SK) and their hybrids (H).

Newhybrid category	Genotype probability		
	SC (AA)	H (AB)	SK (BB)
SC (Parental taxon)	1	0	0
SK (Parental taxon)	0	0	1
F_1 (First-generation hybrid)	0	1	0
$B_1 \times$ SC (First-generation backcross)	0.5	0.5	0
$B_1 \times$ SK (First-generation backcross)	0	0.5	0.5
F_2 (Second-generation hybrid)	0.25	0.5	0.25
$B_2 \times$ SC (Second-generation backcross)	0.75	0.25	0
$B_2 \times$ SK (Second-generation backcross)	0	0.25	0.75
$B_3 \times$ SC (Third-generation backcross)	0.875	0.125	0
$B_3 \times$ SK (Third-generation backcross)	0	0.125	0.875
$B_4 \times$ SC (Fourth −generation backcross)	0.9375	0.0625	0
$B_4 \times$ SK (Fourth −generation backcross)	0	0.0625	0.9375

2.3 Library preparation, gene capture and sequencing

463 SNP loci with a differentiation index of one ($F_{ST} = 1$) between *S. chuatsi* and *S. kneri* were selected from the 16 943 single-copy nuclear gene loci enriched in the Sinipercids (Song et al., 2017) (Supplementary Tab. 1). Gene probes with lengths of 80–120 bp were designed to cover 639 polymorphic sites. The 5′ end of the probe was modified with biotin, which could specifically bind to streptavidin to enrich capture products. First, a high-performance focused ultrasonic crusher Covaris E220 (Covaris, Woburn, USA) was used to fragment the sample DNA. The library was prepared using a DNA library preparation kit (NEB Next Ultra II DNA). The library was detected quantitatively using a qubit 4.0 device, and then 200 ng was taken from each library. A hybridization reaction was mixed for every 8 libraries. A 2 × 150 paired-end sequencing was performed using the Illumina Novaseq 6000 (Illumina, Inc, CA, USA) platform.

2.4 Data analysis and SNP identification

The raw reads obtained by sequencing are removed from the adapter sequence and excluded reads with Phred score less than 20. Use BWA software (Burrows Wheeler Aligner) to align the sequencing data to the target region sequence by pair-end mapping. Then use Samtools software to find out the SNP/Indel site of the target area (Li et al., 2009). First we compare sequencing results of *S. chuatsi* and *S. kneri* and screen out molecular markers that can identify *S. chuatsi* and *S. kneri*. For this filtering step, we selected the three following criteria: (1) no heterozygote alleles presented in both species; (2) no more than four heterozygotes in any one species; and (3) no more than two missing data for both species. After these filtering steps the species-diagnostic SNPs of *S. chuatsi* and *S. kneri* were obtained.

2.5 Genealogical classification

We analysed the genotypes by computing the posterior distribution of individual assignment into hybrid categories by NEWHYBRIDS version 1.1 beta (Anderson and Thompson, 2002). The following classes were set using "Jeffreys-like priors" (Anderson and Thompson, 2002) in NEWHYBRIDS: parental *S. chuatsi* (SC), parental *S. kneri* (SK), F_1 hybrids (parental species × parental species), first-generation back-cross (F_1 hybrid × either parental species), second-generation backcross (first-generation backcross × either parental species), third-generation backcross (second-generation back-cross × either parental species), fourth-generation backcross (third-generation backcross × either parental species) and F_2 hybrids (Tab. 2). All simulated individuals were then blindly reassigned to their most probable category using

Table 3. Assignment of the intermediate forms using Newhybrids.

Newhybrid category	Lake Poyang		Lake Dongting		Number
	N	%	N	%	
SC	8	21.05%	0	0	1, 2, 4, 5, 8, 9, 35, 36
SK	25	65.79%	7	70%	7, 10–16, 18–31, 33, 34, 37, 41–45, 47, 48
F_1	2	5.26%	0	0	17, 32
$B_1 \times SC$	1	2.63%	0	0	6
$B_1 \times SK$	0	0	0	0	
F_2	0	0	0	0	
$B_2 \times SC$	1	2.63%	0	0	38
$B_2 \times SK$	0	0	0	0	
$B_3 \times SC$	0	0	0	0	
$B_3 \times SK$	0	0	0	0	
$B_4 \times SC$	0	0	0	0	
$B_4 \times SK$	1	2.63%	3	30%	3, 39, 40, 46
Total H	5	13.16%	3	30%	
Total	38	100%	10	100%	

NEWHYBRIDS. The software was run for 100 000 iterations in the burn-in, followed by 100 000 Markov Chain Monte Carlo iterations in each analysis (Lamer et al., 2015).

3 Results

3.1 Target gene sequencing

After enrichment and sequencing of the DNA library, an average of 2 576 400 original sequences were generated per sample (reads: 101284–13890574). After trimming and desplicing and quality control, an average of 1225357 sequences were obtained per sample (reads: 41882–10103683). All samples have the 463 targeted loci enriched (Supplementary Tab. S1), with an average coverage depth was of $1000\times$.

3.2 Diagnostic SNPs between *S. chuatsi* and *S. kneri*

Among the sequencing results of *S. chuatsi* and *S. kneri*, one sample (number D29) failed the quality test due to their low initial DNA quality. Therefore, we excluded this individual when analyzing the specific molecular markers of *S. chuatsi* and *S. kneri*. Among the 463 target sequences, 349 target sequences, including 458 diagnostic SNPs loci can be used to distinguish *S. chuatsi* from *S. kneri*. And the data mutation site is consistent with the reference data mutation site. There are multiple diagnostic SNP sites in 106 genes, and one diagnostic SNP site in 224 genes (Supplementary Tab. S2).

3.3 Genetical identification of the "intermediate type"

We selected 224 genes with only one diagnostic SNP (Supplementary Tab. S3) and divided 48 "intermediate forms" into *S. chuatsi* (SC), *S. kneri* (SK), F1 hybrid (B_1), first generation backcross ($B_1 \times SC$), second generation backcross ($B_2 \times SC$), and fourth generation backcross ($B_4 \times SK$) by NEWHYBRIDS 1.1. Among the 38 "intermediate forms" of Lake Poyang, there were 8 *S. chuatsi*, 25 *S. kneri*, 2 F_1 hybrids, 1 first generation backcross ($B_1 \times SC$), 1 second generation

backcross ($B_2 \times SC$) and 1 fourth generation backcross ($B_4 \times SK$). Three fourth generation backcross ($B_4 \times SK$) and 7 *S. kneri* were identified in the "intermediate forms" of Lake Dongting. No other hybridization types was detected.

4 Discussion

4.1 Species-diagnostic markers for detecting hybridization

Microsatellite markers have been successfully used to identify *S. chuatsi* and *S. kneri*, and their hybrids (Tian et al., 2017; Zhu et al., 2020). In comparison to high polymorphism of microsatellite markers, SNPs are more suitable as diagnostic sites due to their low mutation rates and small number of alleles (Balloux and Goudet, 2002). Song et al. (2017) confirmed that both *S. chuatsi* and *S. kneri* were two valid species based on the 4784 SNPs locus. Here, we screened 463 differential genes ($F_{ST} = 1$) that may be used to distinguish between *S. chuatsi* and *S. kneri*. In verifying these markers, we chose some *S. chuatsi* samples from the Amur River, and *S. kneri* samples from the Pearl River in this study, excluding the possibility that these diagnostic markers only existing in a specific geographic area. Finally, 458 effective SNP loci were found to distinguish *S. chuatsi* from *S. kneri*, which were located in 349 gene sequences. At the same time, these markers also provided a sufficient number of diagnostic markers for individual identification of the intermediate morphology specimens and introgressive hybridization between *S. chuatsi* and *S. kneri*.

4.2 Introgressive hybridization between *S. chuatsi* and *S. kneri*

In this study, there were eight hybrid offsprings identified using 224 dignotic SNPs, some whom had complex type of hybrids. When making 6 categories (SC, SK, F_1, F_2, $B_1 \times SC$/ SK) for the classification of hybrid offspring, we only found two F_1 hybrids (17, 32) and two backcross offsprings (6, 38). When based on 12 categories (Tab. 3), we found 38 $B_2 \times SC$

hybirds, and four $B_4 \times$ SK hybrids, the others which were consistent with six hypothesse. When backcross offspring $\times$ backcross offspring, or backcross offspring $\times F_1/F_2$, was adopted to make 26 categories for the hybrid offspring (Lamer et al., 2015). The result was also consistent with 12 categories hypothesis. Therefore, we think that 12 categories hypothesis for the hybrid offspring allocation was suitable for analyzing the samples.

In our study, there were 5 individuals identified as the hybrids in Lake Poyang, categorized as F_1 (2 individuals), $B_1 \times$ SC (1 individual), and $B_2 \times$ SC (1 individual). These types indicated that backcrossing with *S. chuatsi* was more common in the crossing offsprings, and the generations of backcrossing indicated it may be a recent crossing event. At the same time, $B_4 \times$ SK (1 individual) was also found in the crossing offsprings, which indicated that the hybrid could backcrossed with both parent species *S. chuatsi* and *S. kneri*. In the Dongting Lake, only 3 individuals of $B_4 \times$ SK were found, which implied a minor and simple crossing event in this lake. However, this may be due to the small sampling size. In summary, different backcrossing types and generations were found in the intermediate forms from two lakes in the middle reaches of the Yangtze River. Since the hybrid offsprings could backcross with both parent species *S. chuatsi* and *S. kneri*, there was a bidirectional introgressive hybridization.

Introgressive hybridization is one of the main sources of genetic variation in natural populations (Barluenga et al., 2006), which may affect species morphology, behavior, genetics and ecological integrity, and then lead to the reduction of species diversity, the decline of outbreeding and the demise of species (Scribner et al., 2000; Brown et al., 2010). Therefore, studying introgressive hybridization among species (especially homogeneous species) can help us understand the formation and evolutionary mechanisms of species, and help formulate scientific species protection strategies.

Sympatric distribution could provide the possibility of introgressive hybridization between *S. chuatsi* and *S. kneri*, and changes in the ecological environment caused by human activities may accelerate the frequency of introgressive hybridization (Scribner et al., 2000). In recent years, due to climate change, the construction of large hydraulic projects (Three Gorges Dam), overfishing, and lake farming, the hydrological characteristics of the middle reaches of the Yangtze River have changed significantly. Early advances and extending in dry seasons have become the norm for these lakes in the middle reaches of the Yangtze River. In severe cases, only waterways remained in the lake heart areas. These drastic environmental changes may caused fish changing their natural habitats and breeding places, which may be the main reason for the destruction of reproductive isolation between *S. chuatsi* and *S. kneri*, and accidental hybridization in the natural environments. On the other hand, this may also be related to the artificial propagation of mandarin fishes and intensive culturing activities nearing to the Poyang Lake and Dongting Lake. Artificial inter-species hybrids (Lu et al., 2013) maybe escaped into natural water bodies, and crossed naturally with *S. chuatsi* and *S. kneri*. Therefore, it is necessary to strengthen the supervision of propagation and culturing to reduce the potential escapement of the farmed fish.

4.3 Re-examination the morphological characteristics of the intermediate specimens

The detection of morphological characteristics has been used to monitor hybrid individuals and has been assumed to lie between two parent species (Smith, 1992). Lamer et al. (2010) found that the appearance and structure of the gill rakers of bighead carp (*Hypophthalmichthys nobilis*) and silver carp (*H. molitrix*) were indeed useful markers for F_1 generation identification, but they were not 100% accurate, especially for backcross progeny. Based on SNPs identification, it was found that the specimens identified as F_1 hybrids possessed intermediate eye size and humped back, and their maxilla extending to the posterior edge of the eye; while the other backcross specimens showed more similarity to either *S. chuatsi* or *S. kneri* in classification morphology without any regularity.

Among the 48 intermediate specimens, there were also 32 specimens of *S. kneri* and 8 *S. chuatsi* specimens in genetic identification. These *S. kneri* specimens couldn't be distinctly classified because the posterior edge of the maxilla lied directly below the posterior edge of the eyes, whereas *S. chuatsi* specimens were not recognized in classification because of their big eye size. Morphological characteristics of fish are not only determined and formed by genetic factors, but also affected by the environmental effects (Poulet et al., 2004). In addition, mandarin fishes is a ferocious species, and the dramatic difference in feeding intensity in the wild would result in significant variation in growth among individuals (Ren, 1994), and variability of morphological characteristics of individuals within the species in the same lake was prevalent and high (Li et al., 1998). These may accounted for some morphological variations within of *S. kneri* and *S. chuatsi* populations in these lakes.

Supplementary Material

The Supplementary Tables S1 to S3 are available at https://www.alr-journal.org/10.1051/alr/2021001/olm.

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