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Sex-specific markers developed by next-generation sequencing confirmed an XX/XY sex determination system in bighead carp (*Hypophthalmichthys nobilis*) and silver carp (*Hypophthalmichthys molitrix*)

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Abstract

Sex-specific markers are powerful tools for identifying sex-determination system in various animals. Bighead carp (*Hypophthalmichthys nobilis*) and silver carp (*Hypophthalmichthys molitrix*) are two of the most important edible fish in Asia, which have a long juvenility period that can lasts for 4–5 years. In this study, we found one sex-specific marker by next-generation sequencing together with bioinformatics analysis in bighead carp. The male-specific markers were used to perform molecular sexing in the progenies of artificial gynogenetic diploids and found all progenies ($n = 160$) were females. Meanwhile, around 1 : 1 sex ratio was observed in a total of 579 juvenile offspring from three other families. To further extend the male-specific region, we performed genome walking and got a male-specific sequence of 8,661 bp. Five pairs of primers were designed and could be used to efficiently distinguish males from females in bighead carp and silver carp. The development of these male-specific markers and results of their molecular sexing in different populations provide strong evidence for a sex determination system of female homogametry or male heterogametry (XX/XY) in bighead carp and silver carp. To the best of our knowledge, this is the first report of effective sex-specific markers in these two large carp species.

Key words: sex-specific markers, XX/XY sex determination system, bighead carp and silver carp

1. Introduction

The mechanism of sex determination in fish has attracted a lot of biologist's attention, as this mechanism has great implications both in theory and practice.[1] Fish are species of original vertebrate evolved from parthenogenesis to sexual reproduction, and their sex determination systems have showed to be highly diverse and complex.[2–4] Different sex determination systems have been reported in some closely related species or even within the same fish genus.[5] Studies of sex determination in fish provided insights into the genetic mechanisms of sex determination and origin and evolution of sex

chromosomes.[6,7] Many farmed fish exhibit significant sexual dimorphism in body size, growth rate and first time of sexual maturation.[2,8] Therefore, understanding the genetic basis of sex determination is critical for the implementation of breeding and production programs and sex control,[9] and it can greatly add value by culturing monosex populations in some fish species.[6,10] To date, sex determination genes have been identified in few farmed fish species, such as rainbow trout (*Oncorhynchus mykiss*),[11] medaka (*Oryzias latipes*),[12] tiger pufferfish (*Takifugu rubripes*),[13] half-smooth tongue sole (*Cynoglossus semilaevis*)[14] and Nile tilapia (*Oreochromis niloticus*).[15]

Sex-specific markers are prerequisite for understanding the mechanisms of sex determination, identification of sex-determining genes and uncovering genetic architecture related to sex differences.[2,16–18] During the past few decades, different approaches have been used to find sex-specific DNA sequences in aquaculture fishes. Traditional methods such as amplified fragment length polymorphism (AFLP) have been successfully used for the identification of sex-specific markers in more than 20 aquaculture fishes.[10,19–24] With the development of next-generation sequencing (NGS) technologies, many new methods have been developed for screening genes or sex-associated DNA fragments. For example, sex-specifically expressed genes by RNA-seq,[25–29] QTL mapping for sex-linked single nucleotide polymorphism (SNP) markers using high-density SNP linkage maps through restriction site-associated DNA sequencing (RAD-Seq),[30–34] identification of sex-related markers between several sexed individuals using RAD-Seq without generating linkage maps,[35–38] and detecting sex-specific sequences by whole genome sequencing.[14,39–41]

Bighead carp and silver carp not only are important freshwater edible fish native to East Asia but also have been introduced into many other countries for improving water quality and human consumption.[42,43] The global production of bighead carp and silver carp reached 3.4 and 4.9 million tons in 2015, respectively (FAO). The age of first maturation in bighead carp and silver carp normally takes 4 to 5 years or more in central and south China and the incapability of determining the gender before sex maturation has brought troubles for aquaculture. Thus, a sex-specific marker is desirable to discriminate male and female samples at an early life stage of the two carps. The aim of this study is to identify sex-specific markers in the two carps and then apply these sex markers to perform molecular sexing and verification in large number of samples including mature and young fish and progenies from gynogenetic and full-sib families.

2. Materials and methods

2.1. Experimental fish

All experimental procedures about dealing with the fish in this study were approved by the Committee for Animal Experiments of the Institute of Hydrobiology of the Chinese Academy of Sciences, China. The methods used in this study were carried out in accordance with the Laboratory Animal Management Principles of China. The rearing activities of bighead carp in Wuhan, Hubei were approved by the owner of the pond.

A total of 136 sexually matured bighead carp (brood fish) were sampled from the Zhangdu Lake Fish Farm (Wuhan, China), all of which were harvested from the middle Yangtze River and sampled at least 6 years old. The sex phenotype of each sire and dam was individually confirmed by collecting its gametes (sperm or eggs) released in artificial reproduction in May 2014. Artificial gynogenesis was performed in bighead carp according to a previous method.[44] In total, 160 young progenies from three diploid gynogenetic families were sampled after 6-month culture in a pond for the detection of genetic sexes in the gynogenesis of bighead carp. In addition, 579 young progenies from three full-sib families were sampled after 6-month culture for the detection of sex ratio. The 16 silver carp samples used to verify the markers were sampled from Shishou Fish Farm (Shishou, China), and they were caught from the Yangtze River when they were young and then reared in the farm until sexually mature. The sex of these fish was confirmed by collecting gametes released. Fin clips from each of the above mature or young carp were collected and preserved in 95% alcohol at 4 °C. Genomic DNA were extracted from alcohol-preserved fin tissues following a standard phenol–chloroform protocol.[45] The quality and quantity of extracted DNA were checked by NanoDrop2000 spectrophotometer (Thermo Scientific, USA) and 1% agarose gel electrophoresis.

2.2. 2b-RAD sequencing and male-specific tags filtering

To identify potential male-specific sequences in a bighead carp genome, we used a modified 2b-RAD genotyping method to find putative SNP loci that may be associated with each sex. In this study, we constructed 2b-RAD libraries for five sires and five dams of bighead carp with known sex phenotypes (brood fish). A total of 250 ng of genomic DNA from each fish was digested with *Bcg*I restriction enzyme (New England Biolabs, USA) at 37 °C for 4 h. The digestion products were heat-inactivated for 20 min at 65 °C, and then linked to adapter 1 and adapter 2 at 16 °C overnight. The linked fragments were amplified with Phusion High-Fidelity DNA Polymerase (Thermo Scientific, USA) using a set of four primers by which sample-specific barcode and sequencing primers were introduced. After 14 cycles of amplification, the library was obtained by purifying the products at ~170 bp via retrieval from 8% polyacrylamide gels. Each library was pooled with an equal amount to make a final library that was sequenced in a lane of the Illumina HiSeq 2500 SE50 platform (Illumina, USA). Rawdata were deposited in NCBI SRA PRJNA401338.

Reads with low-quality bases were removed before the following analyses were carried out. Consensus tags were constructed for each sample with Stacks 1.31[46] with parameter '-m 4 -p 6 -M 1 -N 2 -d –r'. After that, with Perl scripts, we extracted male-specific tags present in all five sires but not in any of the five dams. The male-specific tags were then mapped to a female bighead carp genome (Shunping He, unpublished data), and those tags failing to align to the female reference genome were considered as putative male-specific tags.

2.3. Re-sequencing and screening of male-specific genomic scaffolds

Because the male-specific tags obtained from 2b-RAD sequencing method were only 32 bp in length, which are too short to design primers for PCR experiment, we sequenced a whole genome of a male bighead carp, Arp7, which was one of the five dams used in the screening of male-specific tags. The sequencing library was constructed with small insert fragments (300–500 bp), and sequenced by Illumina HiSeq 4000 sequencing platform using 150 bp paired-end reads. The raw reads were filtered by removing adaptor sequences, contamination and low-quality reads. Then those filtered reads were mapped to a female bighead carp genome (Shunping He, unpublished data), and SOAPdenovo2[47] (version 2.04) was used to assembly these unmapped reads to male-specific regions. The male-specific 2b-RAD reads were aligned to the male-specific regions, and

the targeted scaffolds were regarded as putative male-specific scaffolds.

2.4. Development and validation of male-specific SCAR markers

Three sequence characterized amplified region (SCAR) markers were designed based on each male-specific scaffolds using Primer Premier 5 (Premier Biosoft International, Palo Alto, CA, USA). Then PCR was used to validate the sex specificity of male-specific SCAR markers. PCR reactions were carried with a total volume of 25 μl, containing 50 ng of template DNA, 2.5 μl of 10× reaction buffer, 0.75 U of Taq polymerase (TaKaRa, Japan), 0.8 μl of dNTP (2.5 mmol/l), 0.8 μl of forward and reverse primer mixture (2.5 μmol/l each) and water to the final volume. PCR programs were as follows: an initial denaturation step of 94 °C for 5 min; then 36 cycles of 94 °C for 35 s, 50 °C annealing for 35 s, and 72 °C for 40 s; and finally an extension step of 72 °C for 8 min. The amplified products were separated by 1% agarose or 8% polyacrylamide gel electrophoresis.

Eight male and eight female matured fish with known sexes were first used to verify the authenticity of SCAR markers by PCR/gel electrophoresis assay. Then PCR products were sequenced via direct Sanger sequencing method to confirm the identity of the nucleotide sequences of sex markers with the sequences of male-specific scaffolds. To further validate that those SCAR markers are truly male-specific, and not simply specific to only several individuals, 120 matured fish (broodstock population) with known phenotypic sexes were used to verify the detection efficiency (accuracy) of those SCAR markers. Moreover, SCAR markers were also verified by 160 gynogenetic diploid progenies of bighead carp from three dams.

2.5. Analysis of sex ratio in full-sib families

The male-specific markers were used to assess sex ratio (female to male) in three full-sib families of bighead carp (6 months old), which contained 90, 166, 324 young offspring, respectively. Chi-square tests were performed to determine the fitness of sex ratio (female/male) to the expected 1 : 1.

2.6. Genome walking

The male-specific contigs did not have corresponding regions in female bighead carp genome (Shunping He, unpublished data), so several rounds of genome walking experiments were carried out to gain flanking sequences of the male-specific contigs. Genome walking was performed using Universal GenomeWalker 2.0 kit (Clontech) in accordance with the user's manual. The primers used in genome walking were summarized in Table 3.

3. Results

3.1. Male-specific 2b-RAD tags

To find sex-specific markers in bighead carp, we used 2b-RAD sequencing to genotype five male and five female samples, which were within the 136 brood fish. In total, 30,941,847 raw reads and 26,732,738 high-quality reads were obtained after filtering low-quality reads (Table 1). A total of 16,137,193 high-quality reads were obtained from five female samples and 10,595,545 high-quality reads from five male samples. Each individual gets 1,367,315 to 5,162,352 high-quality reads, and the average amount of data is 2,673,274 reads per sample. After clustering, the tags per sample ranged from 59,506 to 70,463 with an average of 63,699. Average coverage depth per tag among samples varied from 22.28× to 86.75× (mean 42.69×). The high-sequencing coverage depth guarantees the high reliability of sex-specific sequences generated in this study. With Perl scripts, we extracted those male-specific tags presented in all five males but not in any of five females. Finally, we got 13 tags presented in all five male fish but not in any of five female fish. Reads supporting the 13 tags in all male samples were summarized in Table 2.

Table 1. Summary of 2b-RAD data for 10 bighead carp parents

Parents	Sex	Raw reads	High-quality reads	Tags	Sequencing depth
Arp1	♀	1,528,656	1,433,493	63,696	22.51
Arp2	♀	5,951,567	4,867,677	61,397	79.28
Arp3	♀	2,512,315	2,323,809	70,463	32.98
Arp4	♀	2,564,547	2,349,862	70,263	33.44
Arp5	♀	6,344,744	5,162,352	59,506	86.75
Arp6	♂	1,578,254	1,479,263	64,451	22.95
Arp7	♂	5,911,337	4,852,376	60,226	80.57
Arp8	♂	1,552,576	1,436,797	63,093	22.77
Arp9	♂	1,450,791	1,367,315	61,362	22.28
Arp10	♂	1,547,060	1,459,794	62,534	23.34
Average	♂	3,094,185	2,673,274	63,699	42.69

Table 2. Information of 13 male-specific tags

Name	Tag	Sequence	Existence in the female genome	Scaffold of the male genome
1	ref-22847	AGTAAACAGACGAGCAAACTGCAGCAAAAGAA	No	scaffold37502
2	ref-18178	AAATTGGCAACGAAAAAACTGCAGCGCGTATG	No	scaffold8218
3	ref-804	ACTCCTTTAACGATGAAGATGCAATTTTCGCC	No	—
4	ref-14673	CTGTTGAAGACGATGGAGGTGCGGATGACGTT	Yes	—
5	ref-18221	TGTCTTTTTACGAGACAGATGCTCCAGCATCA	Yes	—
6	ref-20508	ACCAAAGACACGATCACTGTGCTATAGCGTGC	Yes	—
7	ref-44705	CACATGAACCCGAGGGCAATGCATTCTGAATC	Yes	—
8	ref-45942	TCATCAGCATCGAGACTCCTGCCATTTCATTC	Yes	—
9	ref-46385	TGCATATGTCCGATAGGATTGCAACATTCAAA	Yes	—
10	ref-47639	TTATTTTCATCGACAAACTTGCCATTCGAGTC	Yes	—
11	ref-58079	TCTTGGATCCCGAAATAGCTGCCCGGAGGCGT	Yes	—
12	ref-63125	AGATGAGCTCCGAGTTCTGTGCCATCATTGCC	Yes	—
13	ref-68081	GCAACAATCTCGACCGCATTGCCGAAGAGGCC	Yes	—

We mapped these 13 potential male-specific tags to the female bighead carp genome sequence (Shunping He, unpublished data), and found 10 of them were mapped to a female genome. Among them, five had SNPs in the *Bcg*I digestion recognition site (N_{10}CGAN_6TGCN_{10}) of the female genome, and other five tags remained the same sequences as their corresponding female sequences. Therefore, only three tags without any mapping were male-specific 2b-RAD tags (Table 2).

3.2. Mapping male-specific tags to male-specific genomic regions

Since male-specific 2b-RAD tags were too short to design primers for PCR amplification, one of the male individuals was subject to short-reads *de novo* sequencing. We got 48,157,939 pairs of 150 bp Illumina pair-end reads for this sample. After filtering low-quality reads, 42,830,738 pair-end reads were left and mapped to a female bighead carp genome. As mapped reads were located in regions identical between the male and female genome, we only kept 4,417,710 pairs of unmapped reads for *de novo* assembly of male-specific regions of bighead carp. Finally, we got 298,474 scaffolds with a total size of 79.0 Mb.

To get the flanking sequences of the three tags, we mapped them to assembled male-specific scaffolds. For the tag ref-22847, it was mapped to scaffold37502 (907 bp) with 100% identity. For the tag ref-18178, it was mapped to scaffold8218 (602 bp) of identical sequences. For the tag ref-804, we could not find any mapping results in the scaffolds of the male genome. Therefore, scaffold37502 and scaffold8218 were candidate male-specific sequences in the bighead carp.

3.3. Development and validation of male-specific markers

Three primers were designed for each male-specific scaffolds, and then their authenticity was verified by PCR between mature males and females (Table 3, Supplementary File S2). Three markers from scaffold37502 showed consistent amplification of fragments in all eight mature males, while none of the eight mature females amplified those bands (Fig. 1). For scaffold8218, the PCR products from all three primers showed no difference between male and female samples (Supplementary Fig. S3). In order to detect universality of the male-specific markers, the three markers from scaffold37502 were tested in another 120 mature bighead carp fish, and the authenticity of these sexing tests was 100% (Supplementary Fig. S1). These results strongly support high reliability of the male-specific markers in other larger bighead carp samples, which confirmed that these loci are truly male-specific. These male-specific markers were also validated in 160 offspring from three diploid gynogenetic families, and all fish were identified as females (Supplementary Fig. S2). The male PCR products were Sanger sequenced, and the nucleotide sequences were consistent with the male genomic sequences in scaffold37502. However, three markers from scaffold8218 showed no difference when amplified between female and male individuals. Therefore, only scaffold37502 was confirmed as a male-specific sequence in bighead carp.

We blasted the male-specific sequences of scaffold37502 against the female genome of bighead carp, but no homologous sequences were found. Then we blasted the male-specific sequences of the scaffold37502 against the NCBI NT database, and we could not find sequence alignment of this region to any other vertebrate genomes.

Table 3. Male-specific primers used for SCAR markers

Name	Reference sequences	Primers (5′-3′)
ArS-9-1	scaffold37502	F: GCTCCTTACTCAGCAACT
		R: TCAGTAAACAGACGAGCA
ArS-9-2	scaffold37502	F: GGTGCAGGATTTCCAGTT
		R: CCATTGATGTTGTCGCTCT
ArS-9-3	scaffold37502	F: CAAAGACCGCAATAGGAG
		R: GAGCATGTGAAATTAGTGAAG
ArS-9-10	Genome walking	F: GGCTATCTAAGTTTGGGC
		R: GGATGAGCATTGAAGGTG
ArS-9-11	Genome walking	F: GTAAGTTGAGTTTGTGGC
		R: GATGAGCATTGAAGGTG
ArS-9-13	Genome walking	F: CAAAGACCGCAATAGGAG
		R: CCAGGACAAGGTGACATACT
ArS-9-14	Genome walking	F: TCGGCAAACAGAAAAGAC
		R: AATGGTGAATAGGGAGCG
ArS-9-15	Genome walking	F: AGCAACTTTTGTCTGGTG
		R: AATGAATGGTGAATAGGG

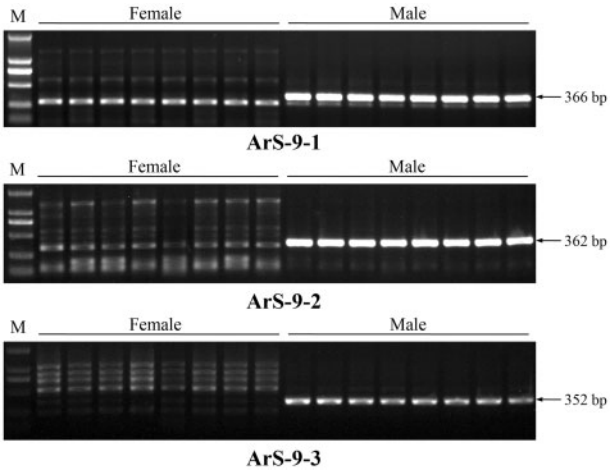

Figure 1. PCR detection and genetic sexing in eight females and eight males of bighead carp by male-specific primer pairs designed based on male scaffold37502. The DL 2000 DNA marker is shown on the left.

The earlier results indicated that these sequences from male-specific region are unique to the bighead carp male genome.

3.4. Genetic sex identification in full-sib families

After verifying the authenticity of male-specific markers, they were used to analyse the ratio of females to males in three full-sib families of young bighead carp containing 579 offspring, and the total segregation ratio of females to males was 295 : 284 (1.04 : 1) (Table 4) in all three families. The female-to-male segregation ratio of these three families were 46 : 44 (1.05 : 1), 82 : 84 (0.98 : 1) and 295 : 284 (1.04 : 1), respectively. Chi-square testing showed that the female-to-male ratio of all three families did not have significant differences with the 1 : 1 separation ratio ($P = 1$).

3.5. Genome walking and more sex-specific markers

After five rounds of genome walking (two for the upstream and three for the downstream sequences), the corresponding male genomic fragments were extended. The designed walking primers and the

Table 4. The male and female ratio for three full-sib families of bighead carp

Family	Number of offspring	Number of females	Number of males	Ratio
1	90	46	44	1.05
2	166	82	84	0.98
3	323	167	156	1.07
Total	579	295	284	1.04

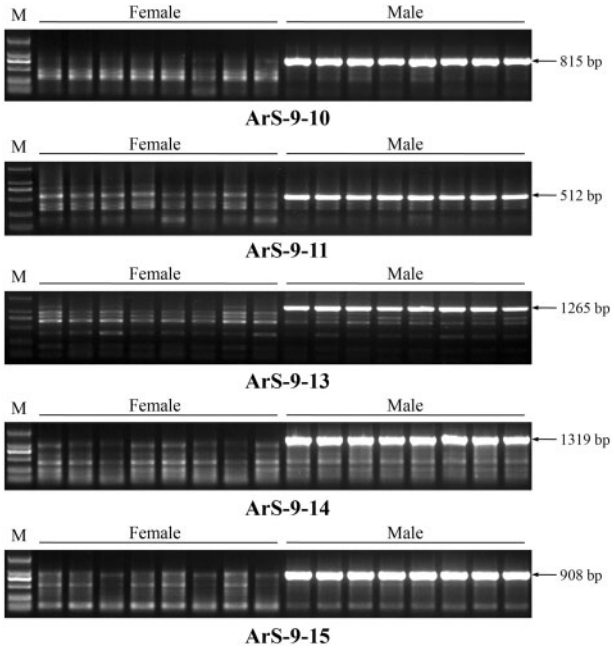

Figure 2. PCR detection and genetic sexing in eight females and eight males of bighead carp by different male-specific primer pairs designed based on genome walking sequences. The DL 2000 DNA marker is shown on the left.

adapter primers were summarized in Table 3. Based on the amplified fragments from males, an 8,661 bp of male-specific sequences were assembled from the male-amplified fragments (Supplementary File S1), and the whole sequence was submitted to NCBI at accession MG668998. According to the male-specific sequences, more sex-specific markers were designed, such as ArS-9-10, ArS-9-11, ArS-9-13, ArS-9-14 and ArS-9-15. The PCR results from different sex with these primers further identified that this region is male-specific region in the bighead carp genome (Fig. 2, Table 3). We blasted the genome walking sequence against the female genome, but no corresponding homologous sequence was found.

3.6. Genetic sex identification in silver carp

In order to verify the presence of the sex markers in other species of the genus *Hypophthalmichthys*, the designed SCAR primers were also amplified in eight female and eight male silver carp and showed the same results with bighead carp (Fig. 3). We also found that the PCR results matched with phenotype with a 100% overall accuracy. The same amplification results were found both on males and females of these two species, and sequences of PCR products of silver carp were also similar with that of bighead carp.

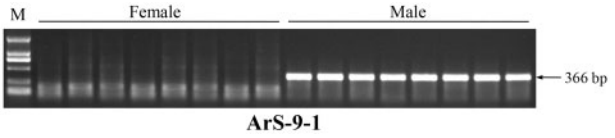

Figure 3. PCR detection and genetic sexing in eight females and eight males of silver carp by male-specific primer pairs designed in bighead carp. The DL 2000 DNA marker is shown on the left.

4. Discussion

Sex-associated markers' identification can facilitate the discovery of sex-determining region or even sex-determining genes. The results can also help us understand the sex-determination systems as well as origin and evolution of sex chromosomes. However, unlike the mammals or birds with significant difference in sex chromosomes, large differentiated genomic regions are uncommon in fish.[48] Thus, it is always difficult to screen these sex-specific regions successfully. Traditional molecular marker technologies such as RAPD and AFLP have proved to be simple and effective ways to find sex markers in fish,[21–24,49] but in some species they failed to detect any sex-specific markers.[50–52]

In the last decade, the development of NGS technology allowed researchers to quickly and conveniently obtain millions of sequences at the genomic level, and it rendered new opportunities for sex difference analysis between males and females.[37,53] With the combination of NGS and restriction digestion enzymes, RAD-Seq offers the possibility for generating thousands of SNPs in a short time, which could be used for identification of sex-linked SNP markers or sex-specific DNA sequences and for the construction of high-density linkage maps and sex QTL mapping.[32,36,38,53] RAD-Seq has been successfully used for sex-specific marker identification in a variety of species using only multiple male and female individuals, which proved to be an efficient way for screening of sex-specific markers.[35–38,53] But RAD-Seq always misses some of the digestion sites, which can cause the failure of identification of sex-specific markers.

Compared to other RAD-Seq methods, the 2b-RAD method used in this study has more advantages compared to traditional RAD-Seq. First, it can screen nearly every restriction site at the whole genome level whereas other RAD-Seq methods can only get a subset of total sites due to the multiple time size selection for efficient PCR amplification and sequencing.[54,55] The average sequence coverage for each site in 2b-RAD is usually more than 40× coverage, which provided accuracy of 2b-RAD genotyping and high reliability of generated tags.[55] Second, the sequences of 2b-RAD are easy for sex-specific 2b-RAD reads identification, which is very convenient for PCR verification. With all of these positive attributes, the 2b-RAD method is very suitable for sex-specific markers and sex-determining regions identification. Here, for the first time, we utilize the 2b-RAD and genome re-sequencing to efficiently and accurately identify the sex-specific markers in genomes of two closely related species, bighead carp and silver carp. Our study proved that combining 2b-RAD genotyping and genome re-sequencing is a very powerful and promising tool for sex-specific markers' identification. This strategy of SCAR-based PCR detection in this study is the first protocol successfully amplifying the Y-specific fragment in bighead carp and silver carp.

The studies of sex-determination in fish would contribute to the understanding of the origin and evolution of sex chromosomes.[1,3] Although most fish species lack visually heteromorphic sex chromosomes, some species have evolved sex-determining regions (genes)

and even nascent sex chromosome.[12,23,30,32,34,38] Based on the 2b-RAD sequencing and male fish re-sequencing, we got a scaffold spanning over 900 bp, which is totally associated with Y-specific fragments of bighead carp, suggesting that this scaffold is located on the Y chromosome or nascent Y-linked region. We extend this scaffold to 8,661 bp by genome walking, but no homologous sequences corresponding to this region was found in the female genome. This might be a significant difference in male and female sex-determining regions in bighead carp. The diversity between male and female fish in this sex-related region may lead to recombination suppression as an early stage of sex chromosome evolution. Our results and previous karyotyping studies demonstrated that bighead carp has evolved a sex-determining region, but has not yet formed distinguishable (heteromorphic) sex chromosomes.[56]

To study whether Cyprinidae fish shared the same sex-determination system, we blasted the 8,661 bp sequences to the male-specific region in grass carp (*Ctenopharyngodon idellus*) genome,[39] and found that most of the 4,148 bp of male-specific sequences in grass carp were presented in bighead carp male-specific regions with ~94% identify. Furthermore, one male-specific marker has been found in common carp (*Cyprinus carpio*) from the Yellow River,[57] but we found that sequence presented in gynogenetic Songpu common carp genome. This demonstrates that this sequence might be a strain-specific sex marker in common carp. And we found no similarity between it and male-specific marker in bighead carp. Unlike the big carp, the sex-determination of zebrafish has been found to be both genetic and environmental.[33] Several markers have been found in the zebrafish genome. Chromosome 4 are closely-related to its sex,[58] but we found the male-specific region in bighead carp cannot mapped to that region in zebrafish genome. The reason for above phenomena might be the ancestor of zebrafish divided with the ancestor of Endemic Clad of East Asia Cyprinidae (ECEAC) fish at about 30 Mya, which is much older than specification of fish within ECEAC (about 5~9 Mya).[59] Based on this information, the bighead carp male-specific sequence was not only shared in silver carp but also in grass carp, and we predict that this male-specific sequence might emerge in the ancestor of ECEAC fish and was kept in all extent diploid fish in this group. As to the common carp, which is a tetraploid fish, it might have formed its own sex-determination region or genes in its speciation process.[60]

Sex-specific markers have been successfully used to identify the sex determination systems in many species,[18,24,35–38] and it is especially valuable in species that lack heteromorphic sex chromosomes.[37] In this study, the presence of male-specific markers indicates an XX/XY sex determination system in bighead carp and silver carp. A larger sample size of bighead carp was used to further verify the accuracy of the male-specific markers, and the verification efficiency was 100% in all mature individuals. Besides, the female-to-male segregation ratio in three full-sib families was 1 : 1. All those results strongly indicated that bighead carp and silver carp have a genetic XX/XY sex determination system, which is similar to the grass carp's XX/XY sex determination system.[39] Based on these results and the phylogenetic position of fishes discussed earlier, we predicted that the ancestor of the whole Cyprinidae might have a XX/XY sex determination system. And this system might persist in all extent species in Cyprinidae and facilitate the hybrid between different species within this group.

Bighead carp and silver carp are two of the most closely related species of the Cyprinidae family, cytogenetic analyses revealed that they have similar karyotypes and lack visually heteromorphic sex chromosomes.[56,61] The linkage groups of bighead carp and silver carp showed a 1 : 1 best orthologues relationship based on comparative genome mapping,[62] meaning a low level of genetic diversity. In this study, bighead carp, silver carp and grass carp shared the same sex-specific markers and sex-determining DNA sequence, suggesting that they may have a homologous nascent Y chromosome and the same pathways involved in sex determination systems. Therefore, the three carp should share the most recent common ancestor and the sex determination systems probably arose before speciation.

We presented an approach that identifies and validates sex-specific markers using 2b-RAD sequencing and genome re-sequencing from multiple male and female individuals in bighead carp and silver carp. A male-specific scaffold was identified and three sex-specific primers were designed first according the male-specific scaffold. The sex-specific markers could be used in genetic sexing with 100% accuracy in brood fish and a large number of young fish from full-sib families. In addition, all gynogenetic progenies were genetically sexed as female by these sex-specific markers, confirming the female homogamety sex determination system in bighead carp. Taken together, all these results confirm that bighead carp and silver carp have a genetic mechanism of sex determination with an XX/XY sex determination system. The sex-specific markers developed in this study would be powerful and effective tools to uncover the sex determination system and identify potential genomic regions in bighead carp and silver carp.

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Accession numbers

PRJNA401338 and MG668998

Conflict of interest

None declared.

Supplementary data

Supplementary data are available at *DNARES* online.

References

1. Devlin, R. H. and Nagahama, Y. 2002, Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences, *Aquaculture*, **208**, 191–364.
2. Kobayashi, Y., Nagahama, Y. and Nakamura, M. 2013, Diversity and plasticity of sex determination and differentiation in fishes, *Sex Dev*., **7**, 115–25.
3. Kikuchi, K. and Hamaguchi, S. 2013, Novel sex-determining genes in fish and sex chromosome evolution, *Dev. Dyn*., **242**, 339–53.
4. Mank, J. E. and Avise, J. C. 2009, Evolutionary diversity and turn-over of sex determination in teleost fishes, *Sex Dev*., **3**, 60–7.
5. Volff, J. N. 2005, Genome evolution and biodiversity in teleost fish, *Heredity (Edinb)*, **94**, 280–94.
6. Mei, J. and Gui, J. F. 2015, Genetic basis and biotechnological manipulation of sexual dimorphism and sex determination in fish, *Sci. China Life Sci*., **58**, 124–36.

7. Ellegren, H. 2011, Sex-chromosome evolution: recent progress and the influence of male and female heterogamety, *Nat. Rev. Genet*., **12**, 157–66.
8. Cnaani, A. and Levavi-Sivan, B. 2009, Sexual development in fish, practical applications for aquaculture, *Sex Dev*., **3**, 164–75.
9. Martínez, P., Viñas, A. M., Sánchez, L., et al. 2014, Genetic architecture of sex determination in fish: applications to sex ratio control in aquaculture, *Front. Genet*., **5**, 340.
10. Wang, D., Mao, H. L., Chen, H. X., Liu, H. Q. and Gui, J. F. 2009, Isolation of Y- and X-linked SCAR markers in yellow catfish and application in the production of all-male populations, *Anim. Genet*., **40**, 978–81.
11. Yano, A., Guyomard, R., Nicol, B., et al. 2012, An immune-related gene evolved into the master sex-determining gene in rainbow trout, *Oncorhynchus mykiss*, *Curr. Biol*., **22**, 1423–8.
12. Matsuda, M., Nagahama, Y., Shinomiya, A., et al. 2002, DMY is a Y-specific DM-domain gene required for male development in the medaka fish, *Nature*, **417**, 559–63.
13. Kamiya, T., Wataru, K., Satoshi, T., et al. 2012, A trans-species missense SNP in Amhr2 is associated with sex determination in the tiger pufferfish, Takifugu rubripes (Fugu), *PLoS Genet*., **8**. DOI: 10.1371/journal.pgen.1002798
14. Chen, S. L., Zhang, G., Shao, C., et al. 2014, Whole-genome sequence of a flatfish provides insights into ZW sex chromosome evolution and adaptation to a benthic lifestyle, *Nat. Genet*., **46**, 253–+.
15. Li, M. H., Yunlv, S., Jiue, Z., et al. 2015, A tandem duplicate of anti-mullerian hormone with a missense SNP on the Y chromosome is essential for male sex determination in Nile tilapia, *Oreochromis niloticus*, *PLoS Genet*., **11**. DOI: 10.1371/journal.pgen.1005678.
16. Gui, J. F. and Zhu, Z. Y. 2012, Molecular basis and genetic improvement of economically important traits in aquaculture animals, *Chinese Sci. Bull*, **57**, 1751–60.
17. Gui, J., Zhou, L. and Wu, Q. 2007, *Genetic Basis and Artificial Control of Sexuality and Reproduction in Fish*. Science Press: Beijing.
18. Charlesworth, D. and Mank, J. E. 2010, The birds and the bees and the flowers and the trees: lessons from genetic mapping of sex determination in plants and animals, *Genetics*, **186**, 9–31.
19. Kovacs, B., Egedi, S., Bartfai, R. and Orban, L. 2000, Male-specific DNA markers from African catfish (*Clarias gariepinus*), *Genetica*, **110**, 267–76.
20. Vale, L., Dieguez, R., Sánchez, L., Martínez, P. and Viñas, A. 2014, A sex-associated sequence identified by RAPD screening in gynogenetic individuals of turbot (*Scophthalmus maximus*), *Mol. Biol. Rep*., **41**, 1501–9.
21. Felip, A., Martinez-Rodriguez, G., Piferrer, F., Carrillo, M. and Zanuy, S. 2000, AFLP analysis confirms exclusive maternal genomic contribution of meiogynogenetic sea bass (*Dicentrarchus labrax L.*), *Mar. Biotechnol*., **2**, 301–6.
22. Felip, A., Young, W. P., Wheeler, P. A. and Thorgaard, G. H. 2005, An AFLP-based approach for the identification of sex-linked markers in rainbow trout (*Oncorhynchus mykiss*), *Aquaculture*, **247**, 35–43.
23. Dan, C., Mei, J., Wang, D. and Gui, J. F. 2013, Genetic differentiation and efficient sex-specific marker development of a pair of Y- and X-linked markers in yellow catfish, *Int. J. Biol. Sci*., **9**, 1043–9.
24. Pan, Z. J., Li, X. Y., Zhou, F. J., Qiang, X. G. and Gui, J. F. 2015, Identification of sex-specific markers reveals male heterogametic sex determination in *Pseudobagrus ussuriensis*, *Mar. Biotechnol*., **17**, 441–51.
25. Lamatsch, D. K., Adolfsson, S., Senior, A. M., et al. 2015, A transcriptome derived female-specific marker from the invasive western mosquitofish (*Gambusia affinis*), *PLoS One*, **10**. DOI: 10.1371/journal.pone.0118214.
26. Lu, J. G., Zheng, M., Zheng, J., et al. 2015, Transcriptomic analyses reveal novel genes with sexually dimorphic expression in yellow catfish (*Pelteobagrus fulvidraco*) brain, *Mar Biotechnol*, **17**, 613–23.
27. Diaz, N. and Piferrer, F. 2015, Lasting effects of early exposure to temperature on the gonadal transcriptome at the time of sex differentiation in the European sea bass, a fish with mixed genetic and environmental sex determination, *BMC Genomics*, **16**. DOI: 10.1186/S12864-015-1862-0.
28. Chen, X., Mei, J., Wu, J., et al. 2015, A comprehensive transcriptome provides candidate genes for sex determination/differentiation and SSR/SNP markers in yellow catfish, *Mar. Biotechnol*., **17**, 190–8.
29. Tao, W. J., Yuan, J., Zhou, L., et al. 2013, Characterization of gonadal transcriptomes from Nile tilapia (*Oreochromis niloticus*) reveals differentially expressed genes, *PLoS One*, **8**. DOI: 10.1371/journal.pone.0063604.
30. Palaiokostas, C., Bekaert, M., Khan, M. G. Q., et al. 2015, A novel sex-determining QTL in Nile tilapia (*Oreochromis niloticus*), *BMC Genomics*, **16**. DOI: 10.1186/S12864-015-1383-X.
31. Palaiokostas, C., Bekaert, M., Taggart, J. B., et al. 2015, A new SNP-based vision of the genetics of sex determination in European sea bass (*Dicentrarchus labrax*), *Genet. Sel. Evol*., **47**. DOI: 10.1186/s12711-015-0148-y.
32. Palaiokostas, C., Bekaert, M., Davie, A., et al. 2013, Mapping the sex determination locus in the Atlantic halibut (*Hippoglossus hippoglossus*) using RAD sequencing, *BMC Genomics*, **14**. DOI: 10.1186/1471-2164-14-566.
33. Anderson, J. L., Marí, A. R., Braash, I., et al. 2012, Multiple sex-associated regions and a putative sex chromosome in zebrafish revealed by RAD mapping and population genomics, *PLoS One*, **7**. DOI: 10.1371/journal.pone.0040701.
34. Palaiokostas, C., Bekaert, M., Khan, M. G. Q., et al. 2013, Mapping and validation of the major sex-determining region in Nile tilapia (*Oreochromis niloticus* L.) using RAD sequencing, *PLoS One*, **8**. DOI: 10.1371/journal.pone.0068389.
35. Carmichael, S. N., Bekaert, M., Taggart, J. B., et al. 2013, Identification of a sex-linked SNP marker in the salmon louse (*Lepeophtheirus salmonis*) using RAD sequencing, *PLoS One*, **8**. DOI: 10.1371/journal.pone.0077832.
36. Kafkas, S., Khodaeiaminjan, M., Guney, M. and Kafkas, E. 2015, Identification of sex-linked SNP markers using RAD sequencing suggests ZW/ZZ sex determination in *Pistacia vera* L., *BMC Genomics*, **16**. DOI: 10.1186/s12864-015-1326-6.
37. Gamble, T. and Zarkower, D. 2014, Identification of sex-specific molecular markers using restriction site-associated DNA sequencing, *Mol. Ecol. Resour*., **14**, 902–13.
38. Fowler, B. L. and Buonaccorsi, V. P. 2016, Genomic characterization of sex-identification markers in *Sebastes carnatus* and *S. chrysomelas* rockfishes, *Mol. Ecol*., **25**, 2165–75.
39. Wang, Y. P., et al. 2015, The draft genome of the grass carp (*Ctenopharyngodon idellus*) provides insights into its evolution and vegetarian adaptation, *Nat. Genet*., **47**, 625–31.
40. Green, J. E., Dalikova, M., Sahara, K., Marec, F. and Akam, M. 2016, XX/XY system of sex determination in the geophilomorph centipede *Strigamia maritima*, *PLoS One*, **11**. DOI: 10.1371/journal.pone.0150292.
41. Koerich, L. B., Dupim, E. G., Faria, L. L., et al. 2016, First report of Y-linked genes in the kissing bug *Rhodnius prolixus*, *BMC Genomics*, **17**, 1.
42. Kolar, C. S., et al. Asian carps of the genus *Hypophthalmichthys* (Pisces, Cyprinidae)-a biological synopsis and environmental risk assessment. 2005.
43. Chen, J., Xie, P., Zhang, D. W. and Lei, H. H. 2007, In situ studies on the distribution patterns and dynamics of microcystins in a biomanipulation fish-bighead carp (*Aristichthys nobilis*), *Environ. Pollut*., **147**, 150–7.
44. Zhu, C. K., Sun, Y. H., Yu, X. M. and Tong, J. G. 2013, Centromere localization for bighead carp (*Aristichthys nobilis*) through half-tetrad analysis in diploid gynogenetic families, *PLoS One*, **8**. DOI: 10.1371/journal.pone.0082950.
45. Chong, L. 2001, Molecular cloning: a laboratory manual, 3rd edition, *Science*, **292**, 446.
46. Catchen, J., Hohenlohe, P. A., Bassham, S., Amores, A. and Cresko, W. A. 2013, Stacks: an analysis tool set for population genomics, *Mol. Ecol*., **22**, 3124–40.
47. Luo, R. B., et al. 2012, SOAPdenovo2: an empirically improved memory-efficient short-read de novo assembler, *Gigascience*, **1**. DOI: 10.1186/2047-217x-1-18.

48. Piferrer, F., Ribas, L. and Diaz, N. 2012, Genomic approaches to study genetic and environmental influences on fish sex determination and differentiation, *Mar. Biotechnol*., **14**, 591–604.
49. Wang, D. W., Li, Y. and Li, Z. Q. 2011, Identification of a male-specific amplified fragment length polymorphism (AFLP) and a sequence characterized amplified region (SCAR) marker in *Eucommia ulmoides* Oliv, *Int. J. Mol. Sci*., **12**, 857–64.
50. Gao, Z. X., Wang, H. P., Yao, H., Tiu, L. and Wang, W. M. 2010, No sex-specific markers detected in bluegill sunfish *Lepomis macrochirus* by AFLP, *J. Fish Biol*., **76**, 408–14.
51. Yarmohammadi, M., Pourkazemi, M., Ghasemi, A., Hassanzadeh, M. and Chakmehdouz, F. 2011, AFLP reveals no sex-specific markers in Persian sturgeon (*Acipenser persicus*) or beluga sturgeon (*Huso huso*) from the southern Caspian Sea, Iran, *Progr. Biol. Sci*., **1**, 55–114.
52. Sriphairoj, K., Na-Nakorn, U., Brunelli, J. P. and Thorgaard, G. H. 2007, No AFLP sex-specific markers detected in *Pangasianodon gigas* and *P. hypophthalmus*, *Aquaculture*, **273**, 739–743.
53. Gamble, T., Coryell, J., Ezaz, T., et al. 2015, Restriction site-associated DNA sequencing (RAD-seq) reveals an extraordinary number of transitions among gecko sex-determining systems, *Mol. Biol. Evol*., **32**, 1296–1309.
54. Baird, N. A., et al. 2008, Rapid SNP discovery and genetic mapping using sequenced RAD markers, *PLoS One*, **3**. DOI: 10.1371/journal.pone.0003376
55. Wang, S., Meyer, E., McKay, J. K. and Matz, M. V. 2012, 2b-RAD: a simple and flexible method for genome-wide genotyping, *Nat. Methods*, **9**, 808–10.
56. Kong, Q. L., LI, Z. Y., Fu, M. L., Wang, Q. and WANG, H. Y. 2006, Analysis of DAPI karyotype of bighead carp (*Aristichthys nobilis*) chromosomes, *Sichuan J. Zool*., **25**, 64–7.
57. Chen, J., Wang, Y., Yue, Y., et al. 2009, A novel male-specific DNA sequence in the common carp, *Cyprinus carpio*, *Mol. Cell. Probes*, **23**, 235–9.
58. Howe, K., Clark, M. D., Torroja, C. F., et al. 2013, The zebrafish reference genome sequence and its relationship to the human genome, *Nature*. **496**, 498–503.
59. Tao, W., Zou, M., Wang, X., et al. 2010, Phylogenomic analysis resolves the formerly intractable adaptive diversification of the endemic clade of east Asian Cyprinidae (Cypriniformes), *PLoS One*, **5**, e13508.
60. Xu, P., Zhang, X., Wang, X., et al. 2014, Genome sequence and genetic diversity of the common carp, *Cyprinus carpio*, *Nat. Genet*. **46**, 1212–19.
61. Su, Z. G., Xu, K. S., Chen, S. P. and Bai, G. D. 1984, Studies on triploid silver carp and its karyotype, *Zool. Res*. **1984**(S2), 15–20.
62. Zhu, C. K., Tong, J. O., Yu, X. M. and Guo, W. J. 2015, Comparative mapping for bighead carp (*Aristichthys nobilis*) against model and non-model fishes provides insights into the genomic evolution of cyprinids, *Mol. Genet. Genomics*, **290**, 1313–26.