

## Research Paper

# Genetic Differentiation and Efficient Sex-specific Marker Development of a Pair of Y- and X-linked Markers in Yellow Catfish

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## Abstract

*Pf62-Y* and *Pf62-X* is a pair of allelic Y chromosome-linked and X chromosome-linked markers, and have been used to identify YY super-males, XY males and XX females for commercial production of all-male populations in yellow catfish (*Pelteobagrus fulvidraco*). However, the SCAR primers used previously have only two nucleotide difference, which restricts the wide utility because of nucleotide polymorphism. In this study, a continuous 8102 bp *Pf62-Y* sequence and a 5362 bp *Pf62-X* sequence have been cloned by genome walking, and significant genetic differentiation has been revealed between the corresponding X and Y chromosome allele sequences. Moreover, three pairs of primers were designed to efficiently identify YY super-males, XY males and XX females in an artificial breeding population, and to distinguish XY males and XX females in various wild populations. Together, the three new sex-specific genetic markers develop a highly stable and efficient method for genetic sex identification and sex control application in sustainable aquaculture of all-male yellow catfish.

Key words: Yellow catfish; Sex-specific marker; genome walking; YY super-males; All-male fish.

## Introduction

Sex-specific or sex chromosome-linked markers have significant implications for genetic improvement of economically important traits in some aquaculture fishes [1][2] because of the uncommon growth difference between females and males [3][4][5]. Previously, male- or female-specific DNA markers were isolated from several important aquaculture fishes, such as African catfish *Clarias gariepinus* [6], Asian arowana (*Scleropages formosus*) [7], Nile tilapia *Oreochromis niloticus* [8][9][10], salt tolerant tilapia hybrid (*Oreochromis mossambicus* × *Oreochromis spp.*) [11], rainbow trout *Oncorhynchus mykiss* [12], half-smooth tongue sole *Cynoglossus semilaevis* [13][14][15], yellow catfish *Pelteobagrus fulvidraco* [16], common carp *Cy-*

*prinus carpio* [17][18], *Paramisgurnus dabryanus* [19], *matrinchā Brycon amazonicus* [20], *Scophthalmus maximus* [21], blunt snout bream *Megalobrama amblycephala* [22], and rock bream *Oplegnathus fasciatus* [23]. In yellow catfish (*Pelteobagrus fulvidraco*), Wang et al. [16] identified two pairs of Y chromosome- and X chromosome-linked SCAR markers by AFLP screening from an artificial breeding population [24], and thereby developed a Y- and X-specific allele marker-assisted sex control technique for mass production of all-male population. Because adult yellow catfish males are about two to three fold bigger than the females, the all-male population, as a novel variety "yellow catfish all-male No. 1", has been widely used

for commercial production throughout China since 2010 [1][25].

Significant implications and application values of the all-male yellow catfish production have been evaluated [1][25][26]. However, these sex-linked SCAR markers used in all-male production were only tested in one artificial breeding population as reported, and the primers of *Pf62-X* and *Pf62-Y* have only two nucleotide difference [16]. In order to search for more accurate molecular markers for large-scale all-male production of yellow catfish, we performed genome walking and sequencing of the Y chromosome-linked marker *Pf62-Y* and X chromosome-linked marker *Pf62-X*, and analyzed the nucleotide divergence and genetic differentiation. Based on these studies, we developed three pairs of new markers that can efficiently identify the genetic sex of yellow catfish in breeding and wild populations.

## Materials and Methods

### Source of experimental samples

The artificial breeding yellow catfish including XX females, XY males and YY super-males came from Institute of Hydrobiology, Chinese Academy of Sciences and from Hubei Daming Aquatic Science-Technology Company (Jingzhou). The wild populations were collected from Liangzi Lake (Wuhan), Chang Lake (Jingzhou), Hong Lake (Honghu), South Lake (Zhongxiang) and Dongting Lake (Hunan).

### Genomic DNA extraction

The genomic DNA was extracted from fin clips of the XX female, XY male and YY super-male individuals using a standard phenol-chloroform method [27], and the quality and concentration of DNA were assessed by ethidium bromide-stained 0.7% agarose

gel under ultraviolet light and by spectrophotometer as described previously [28].

### Genome walking

BD Genome Walker™ Universal Kit (Clontech) was used to construct the DNA fragment pools. High quality genomic DNA sample extracted from XX female and YY super-male was divided into four portions, and four kinds of restriction enzymes: *Dra* I, *Eco*R V, *Pvu* II, *Stu* I were used to digest the DNA samples for 20 h respectively. Phenol/chloroform and 80% ethanol were used to precipitate and rinse the samples, and the digested genomic DNA was dissolved in 1xTE buffer (10 mM Tris, 0.1 mM EDTA). Each batch of digested genomic DNA was then ligated separately to the adapter sequence and used as templates. The adapter primers AP1, AP2 and walking primers listed in Table 1 were designed and synthesized. The genomic walking PCR programs were referred to the manual proposal as described previously [29][30].

The amplified products were examined by 1.0% agarose gel electrophoresis. Single and clear major bands (>500bp long bands) were extracted and purified from gel, and directly sequenced in both directions [31][32]. Sequence fragments were assembled by DNAMAN software.

To confirm the assembled sequence, a pair of primers, 62F (5'-GATCTGGTGACCTACAAAATTGACA-3') and 62R (5'-AAGCACGACTGGGCCAATGTTCCATC-3') was designed according to both ends of the entire sequence. L-A Taq polymerase (Cat.No.A0901A, Takara) was used for amplification reaction through the following PCR program: denaturation in 94°C for 30 seconds, annealing and extension at 68°C for 9 minutes, 36 cycles. The PCR products were detected and purified by 0.8% agarose gel electrophoresis, and sequenced as described previously [33].

**Table 1.** Genomic walking primers for cloning Pf62 markers in yellow catfish

left fragment primers(5'-3')	Right fragment primers(5'-3')
GSP-1a: CTCTGTGCGACACGGGGAACGGTGAAACCT	GSPr-1a: CATAAGCCCTGGGGTAAGATGTCTCTCGGA
GSP-1b: CCGAGAGACATCTTACCCAGGGCTTATGG	GSPr-1b: AGGTTTCACCGTTCCTCCGTCGACAGACA
GSP-2a: TCAAGATGTAAGATGCCTCCCTTCTCGCT	GSPr-2a: TCTCCCTCAAGCTGTTGTCCTTCAAGATG
GSP-2b: GCTTTGATCAGGTTGCTGGATTGCGATGT	GSPr-2b: AACGTGTCGGTGATTTGCATGCGCTCTC
	GSPr-3a: GAGCTGTATGCTGGTCTAATAAACACAT
	GSPr-3b: CTCCTTGCTCAGTGGCGAAGACATCA
	GSPr-4a: CCCTTTTTTCCTTGGGGCTCAAATGAGTC
	GSPr-4b: CCTTACAAATGAAGGTTGATGTTGAAGTTG
	GSPr-5a: AGATGTAAAGGAGGCAGGTTAGCTGCTG
	GSPr-5b: AAAAAGGAGGGGTGGATAAGAAGAGGAC
Adaptor primers:	
AP1: GTAATACGACTCACTATAGGGC	
AP2: ACTATAGGGCACGCGTGGTC	

## Genetic sex identification in yellow catfish

Genomic DNA was respectively extracted from fin clips of the artificial breeding and wild groups yellow catfish. Then, the genomic DNA of each sample was used for PCR amplification to detect male/female genotype by specific primers designed from the genomic walking sequence. Further, the physical sex of yellow catfish was determined by anatomical dissection.

## Results

### Genomic walking and sequence analysis of Y-linked marker *Pf62-Y*

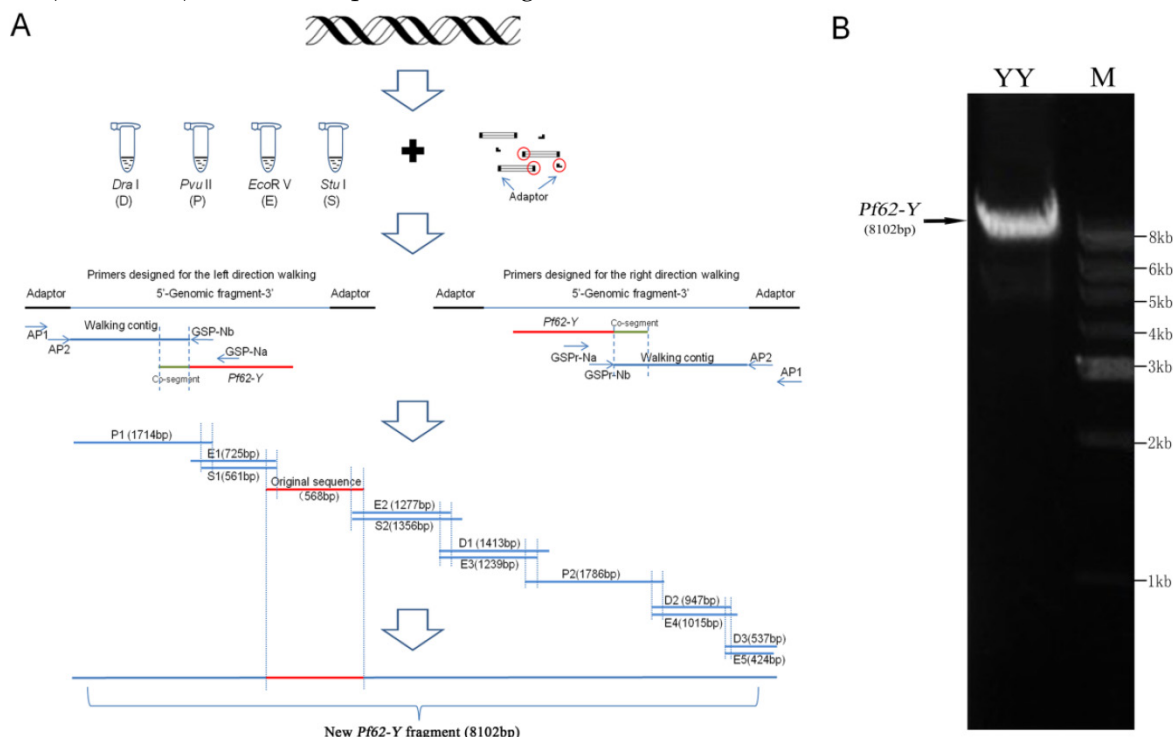
Fig.1A summarized the genomic walking process for *Pf62-Y* sequence. Firstly, the genomic DNA extracted from YY super-male was digested with 4 different restrict enzymes such as *Dra* I, *Pvu* II, *EcoR* V and *Stu* I, and the digested fragments were ligated to adaptor respectively to construct four fragment pools. Then, the designed walking primers and adapter primers were used to amplify the corresponding genomic fragments from the constructed four DNA fragment pools, and the amplified fragments were sequenced. A total of 12 fragments were distinguished. As shown in Fig. 1A, three fragments (D1, D2 and D3) were amplified from the *Dra* I digested DNA fragment pool, two (P1 and P2) from the *Pvu* II pool, five (E1, E2, E3, E4 and E5) from *EcoR* V pool, and two (S1 and S2) from *Stu* I pool. The longest

fragment (P2) contained 1786 bp, and the shortest (E5) was only 424 bp. These walking fragment sequences and the original *Pf62-Y* sequence were analyzed by DNAMAN software and assembled into a continuous 8102 bp *Pf62-Y* fragment (GenBank accession number: KF593811).

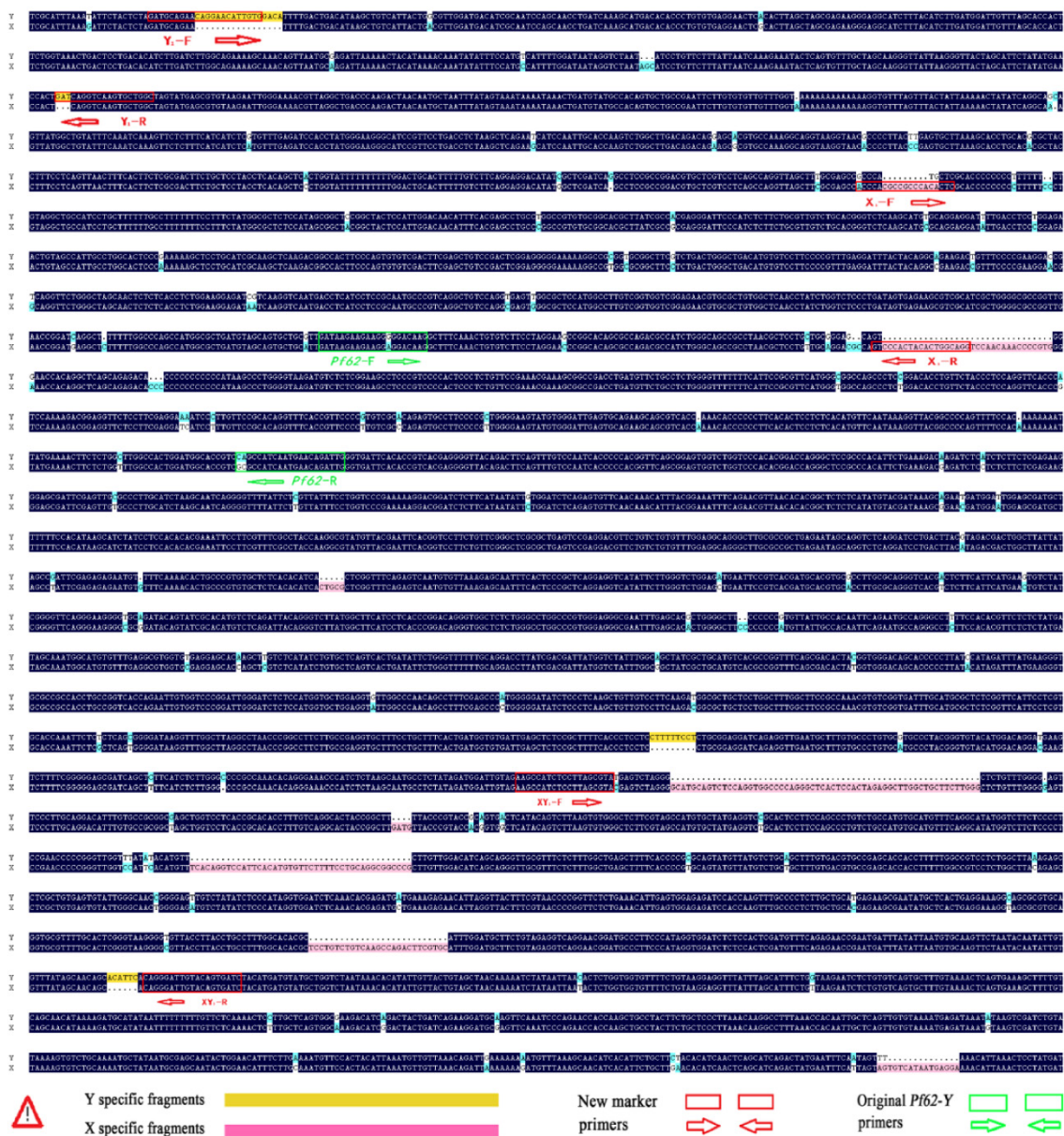
Moreover, an 8102 bp fragment was amplified from an YY super-male by PCR using L-A Taq polymerase (Fig.1B), and its sequence was identical to the assembled sequence from the walking fragments, which confirmed the genomic walking correction.

### Genetic differentiation between *Pf62-Y* and *Pf62-X* allele sequences

To reveal genetic difference between *Pf62-Y* and *Pf62-X* alleles, we further obtained a 5362 bp *Pf62-X* allele sequence (GenBank accession number: KF593812) from a XX female individual by genomic walking and PCR amplification using degenerate primers. Sequence alignment of the 5362 bp *Pf62-X* allele and the corresponding *Pf62-Y* allele shows that the nucleotide identity between them is 87.36%, implicating that there exists substantial genetic differentiation between the *Pf62-X* and *Pf62-Y* alleles (Fig.2). Interestingly, besides a large number of single nucleotide differences, *Pf62-Y* allele has some small segment deletion in comparison with the corresponding *Pf62-X* allele sequence. It seems to imply that the yellow catfish Y chromosome might be highly differentiated during the sex chromosome evolution process.



**Fig 1.** Genomic walking process and sequence confirmation of Y-linked marker *Pf62-Y*. **(A)** The schematic diagram of genomic walking process for *Pf62-Y* sequence. **(B)** Detection of the 8102 bp *Pf62-Y* fragment amplified from an YY super-male in agarose gel.



**Fig 2.** Sequence alignment between *Pf62-Y* and *Pf62-X* alleles. There are numerous single nucleotide differences and some small fragment deletion differences between *Pf62-Y* and *Pf62-X*.

### Design of three pairs of new markers and genetic sex identification in XX females, XY males and YY super-males

Based on the significant genetic difference between *Pf62-Y* and *Pf62-X* allele sequences, we further designed three pairs of new sequence-characterized primers, such as Y chromosome-specific primer pair *Y<sub>1</sub>-F* and *Y<sub>1</sub>-R*, X chromosome-specific primer pair *X<sub>1</sub>-F* and *X<sub>1</sub>-R*, as well as Y and X chromosomes-shared primer pair *XY<sub>1</sub>-F* and *XY<sub>1</sub>-R* (Fig. 2), to identify YY super-males, XY males, and XX females. As shown in Fig. 3, a Y chromosome-specific 398 bp fragment is amplified by the primer pair of *Y<sub>1</sub>-F* (GATGCAGAAGCGAACATTGTG) and *Y<sub>1</sub>-R*

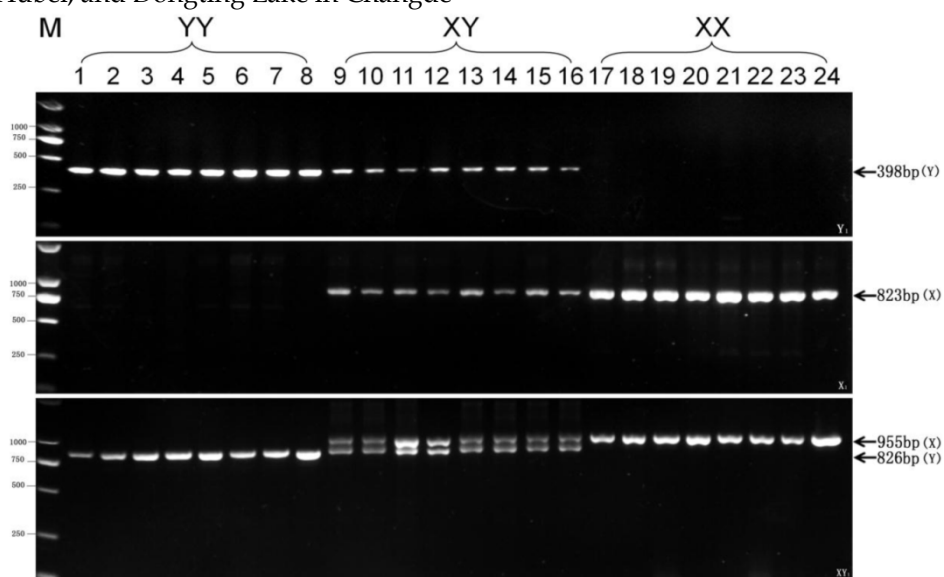
(GCCACCACTTGACCTGATC) from all the YY super-males and XY males, whereas no any product is observed from the XX females. An X chromosome-specific 823 bp fragment is produced by the *X<sub>1</sub>-F* (ACCCACGCCGCCCACTC) and *X<sub>1</sub>-R* (ACCTGCCAGTGTAGTGGGAC) primer pair from XX females and XY males, while no any amplified sequence is obtained from the YY super-males. Owing to the existence of 4 fragment deletions on the *Pf62-Y* allele, two different size fragments (X-fragment: 955bp, Y-fragment: 826bp) are amplified by the primer pair of *XY<sub>1</sub>-F* (GATGTGAGAAGCCATCTCCTTAGCGTA) and *XY<sub>1</sub>-R* (CATGTAGATCACTGTACAATCCCTIG) from the XY males, while only 955bp X-fragment is observed in XX females, and only 826bp

Y-fragment in YY super-males. Therefore, three pairs of new genetic markers ( $Y_1$ ,  $X_1$  and  $XY_1$ ) were developed to efficiently identify YY super-males, XY males and XX females, and the sex determination mechanism ( $XX♀/XY♂$ ) of yellow catfish was further proofed by the X- and Y-specific markers.

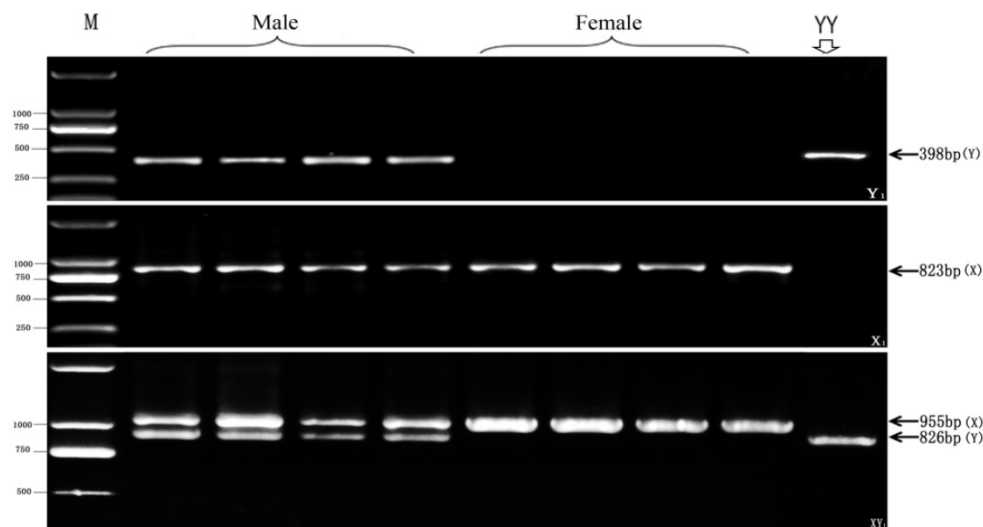
### Genetic sex identification of new markers in 5 different wild populations of yellow catfish

In order to test detection efficiency and applicable scope of the three new genetic markers, we collected different wild population samples of yellow catfish from 5 segregate lakes, such as Liangzi Lake in Wuhan of Hubei, Chang Lake in Jingzhou of Hubei, Hong Lake in Honghu of Hubei, South Lake in Zhongxiang of Hubei, and Dongting Lake in Changde

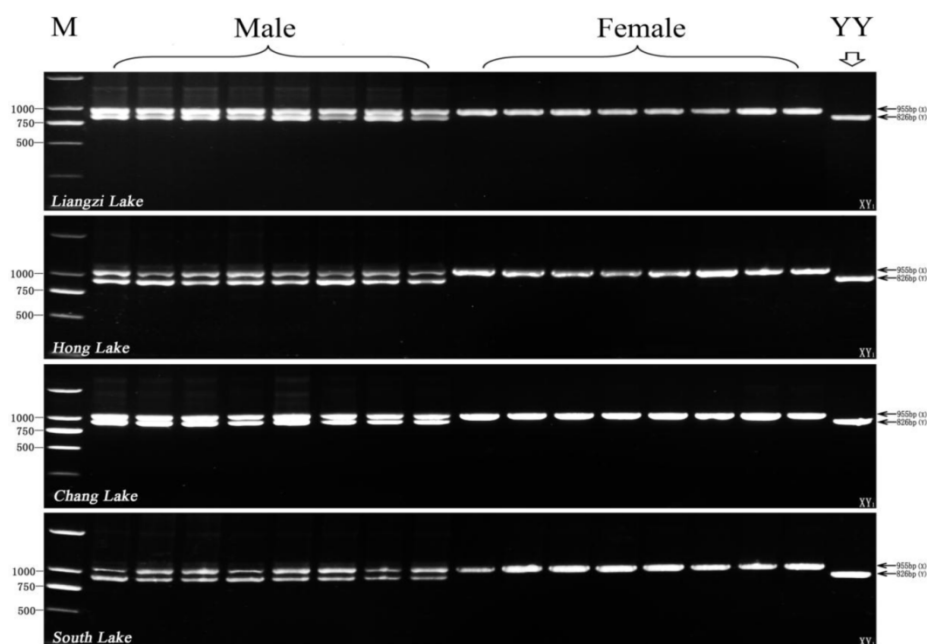
of Hunan. The extracted genome DNAs were respectively used to identify genetic sex and their female or male phenotypes were affirmed by anatomizing ovary or testis. Fig. 4 shows the test results of three new genetic markers in Dongting Lake population samples. Consistent with the detection data in artificial breeding population, a Y chromosome-specific 398 bp fragment is amplified by the primer pair of  $Y_1$ -F and  $Y_1$ -R only in male individuals, whereas an X chromosome-specific 823 bp fragment is detected by the  $X_1$ -F and  $X_1$ -R primer pair in both of females and males. And, two different size fragments (X-fragment: 955bp, Y-fragment: 826bp) are amplified by the primer pair of  $XY_1$ -F and  $XY_1$ -R from the XY males, while only 955bp X-fragment is observed in XX females.



**Fig 3.** Test results of three pairs of new markers ( $Y_1$ ,  $X_1$  and  $XY_1$ ) in 8 YY super-males, 8 XY males, and 8 XX females respectively. The 398 bp Y-specific fragment amplified by  $Y_1$  primer pair, the 823bp X-specific fragment by  $X_1$  primer pair, as well as 955 bp X-specific fragment and 826 bp Y-specific fragment by  $XY_1$ , are indicated by arrows on the right. The DL2000 DNA marker sizes are shown on the left.



**Fig 4.** Test results of three pairs of new markers ( $Y_1$ ,  $X_1$  and  $XY_1$ ) in 4 phenotypic male and 4 female individuals from Dongting Lake wild population. The 398 bp Y-specific fragment amplified by  $Y_1$  primer pair, the 823bp X-specific fragment by  $X_1$  primer pair, as well as 955 bp X-specific fragment and 826 bp Y-specific fragment by  $XY_1$ , are indicated by arrows on the right. The DL2000 DNA marker sizes are shown on the left. YY individual is the control for the YY super-male.



**Fig 5.** Test results of the  $XY_1$  marker in other four wild populations (8 male and 8 female individuals from each population) from Liangzi Lake (Wuhan), Hong Lake (Honghu) Chang Lake (Jingzhou), and South Lake (Zhongxiang). The 955 bp X-specific fragment and 826 bp Y-specific fragment amplified by  $XY_1$  primer pair are indicated by arrows on the right. The DL2000 DNA marker sizes are shown on the left. YY individual is the control for the YY super-male.

Furthermore, we used  $XY_1$ -F and  $XY_1$ -R primer pair to test four other wild populations sampled from Liangzi Lake, Hong Lake, Chang Lake, and South Lake. As shown in Fig. 5, two different size fragments (X-fragment: 955bp, Y-fragment: 826bp) are amplified by the primer pair of  $XY_1$ -F and  $XY_1$ -R from all of the male individuals in 4 wild populations, and only 955bp X-fragment is detected from all of the four wild population female samples. Altogether, the three pairs of new genetic markers ( $Y_1$ ,  $X_1$  and  $XY_1$ ) can efficiently use genetic sex identification of yellow catfish in all sampled populations, and their detection efficiency reaches to 100%.

## Discussion

In this study, we have cloned and sequenced the Y chromosome-linked marker *Pf62-Y* 8102 bp fragment by genome walking, and also obtained its corresponding 5362 bp *Pf62-X* allele sequence. Their corresponding sequence alignment reveal significant genetic difference between them, implicating that significant genetic differentiation has occurred between the corresponding X and Y chromosome regions. It should be a significant finding for this study. In theory, sex chromosome pair has been proposed to evolve from recombination suppression of a pair of autosomes, and the recombination suppression leads to further genetic differentiation and even hetromorphic sex chromosomes [34]. Recently, high degree of sex chromosome differentiation has been also revealed in stickleback fishes by using a set of mi-

cro-satellite markers [35].

Our previous study has demonstrated that the markers *Pf62-Y* and *Pf62-X* are respectively linked to Y chromosome and X chromosome, and they can be used to identify XX females, XY males and YY super-males [16], but the used SCAR primers have only two nucleotide difference, which restricts the wide utility because of nucleotide polymorphism. The genetic difference between *Pf62-Y* and *Pf62-X* allele sequences directly reflects genetic differentiation between Y chromosome and X chromosome. According to the significant genetic difference between *Pf62-Y* and *Pf62-X* allele sequences revealed by the current study, we have designed three pairs of sex-specific primers, and thereby developed three pairs of new genetic markers, such as  $Y_1$ ,  $X_1$  and  $XY_1$ . It has been demonstrated that the three pairs of new genetic markers can efficiently identify YY super-males, XY males and XX females in artificial breeding populations and can efficiently distinguish XY males and XX females in different wild populations of yellow catfish. In summary, our molecular markers highlight a method for sustainable aquaculture of all-male yellow catfish in China.

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## Competing Interests

The authors have declared that no competing interest exists.

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