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Discovery of a male-biased mutant family and identification of a male-specific SCAR marker in gynogenetic gibel carp *Carassius auratus gibelio*

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Abstract

Gibel carp (*Carassius auratus gibelio*) is a uniquely gynogenetic species with a minor ratio of males in natural habitats, but its male origin and sex determination mechanisms have been unknown. In this study, a male-biased mutant family was discovered from the gynogenetic gibel carp, and a male-specific SCAR marker was identified from the mutant family. Normal spermatogenesis was observed in the male testes by immunofluorescence histochemistry. Nearly identical AFLP profiles were observed between males and females, but a male-specific 86 bp AFLP fragment was screened by sex-pool bulked segregant analysis and individual screening. Based on the male-specific AFLP fragment, a total of 579 bp sequences were cloned by genome walking. Subsequently, a male-specific SCAR marker was designed, and the male-specific DNA fragment was confirmed to be steadily transmitted to the next generation and consistently detected only in males.
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Keywords: Gibel carp (*Carassius auratus gibelio*); Gynogenesis; Male-biased mutant family; Male-specific SCAR marker (MSM)

1. Introduction

Since the first unisex fish, the Amazon molly *Poecilia formosa*, was found by Hubbs and Hubbs [1], unisexual species that are composed of all-female individuals have been reported in about 80 taxonomic units of lower vertebrates, of which about 30 are unisexual fish species [2,3]. These unisexual species have been demonstrated to reproduce by gynogenesis, hybridogenesis, or parthenogenesis [4]. And the heterologous sperm effect, subgenomic incorporation from a bisexual related species, or the kleptogenesis reproduction mode have been reported to compensate for disadvantages of unisexual reproduction in the unisex species [5–8].

Gibel carp (*Carassius auratus gibelio*) is a gynogenetic cyprinid fish, which distributes over a large area of Europe and Asia [5,9]. In comparison with other gynogenetic vertebrate species, the gibel carp seems to be more interesting and unique. It possesses several interesting characteristics such as the existence of male individuals in natural habitats [5,10], dual reproduction modes of gynogenesis and sexual reproduction [11], the effect of allogynogenesis [5,12,13] and polyploidy [14,15], which make it an attractive model for sexual evolution and developmental biology in vertebrates [16–19].

In natural habitats, gibel carp was originally described as a bisexual population with a minor but significant portion (5–20%) of males [5,10]. Moreover, artificial propagation experiments, cytological investigation, and molecular marker analysis revealed the dual reproduction modes of gynogenesis and sexuality in gibel carp [11,20]. Hence, in

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artificial propagations, all-female gynogenetic gibel carp have been obtained by inseminating with the sperm of related species such as red common carp (*Cyprinus carpio*) to initiate gynogenesis, and bisexual progeny with a male ratio of about 20% has been produced from crosses between the female and male gibel carp. However, the male origin and sex determination mechanisms remain unknown in gibel carp.

Interestingly, a male-biased mutant family was discovered from the progeny of a clone D gibel carp produced by gynogenesis in 2006 at our experimental station. Generally, in gibel carp, the gynogenetic progenies are composed of all-females, whereas the male-biased mutant family contains 97.2% males. The unexpected mutant family provides a good chance for us to isolate the male-specific genetic marker because the gynogenetic individuals have identical genetic background, and thereby, it is easy to screen their genetic differences between males and females. Based on the discovery of a male-biased mutant family, we attempt to isolate and identify male-specific genetic markers in gynogenetic gibel carp. Here, we report the data.

2. Materials and methods

2.1. Fish source and artificial propagation

Gibel carp used in this experiment came from the Institute of Hydrobiology, Chinese Academy of Sciences. The male-biased mutant family was discovered in 2006 from a clone D progeny of gibel carp produced by gynogenesis stimulated with red common carp sperm. The male-biased mutant family and its following generation were sampled and analyzed in this study. Before sexual maturity, females and males were judged by observing their gonads, and the mature testes were further distinguished by immunofluorescence histochemistry (described below). In the propagation season, the females and males were judged by the ovulated eggs or sperm. To confirm the genetic sex of the rare females (D1♀) in the mutant family, we further propagated the sexual progeny by inseminating the eggs with the sperm from the mutant male and gynogenetic progeny by inseminating the eggs with red common carp sperm. Subsequently, their sexes were distinguished by examining gonads when reaching sexual maturity, and their fin clips were sampled for genetic analysis. Additionally, all the males and females were demonstrated to be triploid by Phoenix Flow Systems according to the method of Wei et al. [21].

2.2. Immunofluorescence histochemistry

The RNA helicase *Vasa* gene is a germ cell marker in animals, and the homolog gene *Cagvasa* has been identified from the gibel carp by Xu et al. [18]. During spermatogenesis, expression of the *Vasa* gene was found to be abundant in spermatogonia and spermatocytes, low in spermatids, and absent in sperm. In combination with a nuclear dye, the CagVasa antibody can differentiate different spermatogenic cells and sperm [18]. Therefore, in this study, the anti-CagVasa immunofluorescence histochemistry was used to trace the process of spermatogenesis in the mutant males and the progeny. The immunofluorescence histochemistry was conducted as described previously [18].

2.3. Genomic DNA extraction and AFLP analysis

Genomic DNA was extracted from fin clips of all the sampled fish using a standard phenol–chloroform method, and the quality and concentration of DNA were assessed by ethidium bromide-stained 0.7% agarose gel under ultraviolet light and by the spectrophotometer (Eppendorf). The AFLP was conducted as described previously [22] following five steps: (1) restriction of DNA by two enzymes, Eco-

Table 1
AFLP adaptors and primers used in this study.

Adaptors and primers	Sequences			
EcoRI adaptor	5′-CTC GTA GAC TGCGTA CC-3′ 3′-CAT CTG ACG CATGG TTAA-5′			
MseI adaptor	5′-GACG ATG AGTCCT GAG-3′ 3′-TAC TCA GGA CTCAT-5′			
EcoRI + 1 primer	5′-GACTGCGTACCAATTCA-3′			
EcoRI + 3 primers	5′-GACTGCGTACCAATTCANN-3′			
	E1-AAC	E5-ACC	E9-AGA	E13-AAA
	E2-AAG	E6-ACG	E10-AGT	E14-AAT
	E3-ACA	E7-AGC	E11-ATC	E15-ATA
	E4-ACT	E8-AGG	E12-ATG	E16-ATT
MseI + 1 primer	5′-GATGAGTCCTGAGTAAC-3′			
MseI + 3 primers	5′-GATGAGTCCTGAGTAACNN-3′			
	M1-CAA	M5-CTA	M9-CCA	M13-CGA
	M2-CAC	M6-CTC	M10-CCG	M14-CGT
	M3-CAG	M7-CTG	M11-CCT	M15-CGC
	M4-CAT	M8-CTT	M12-CCC	M16-CGG

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RI and MseI, and simultaneous ligation of adaptors, (2) amplification of the resulted fragments with preselective primers (with one selective nucleotide), (3) amplification of the diluted products with selective primers (three selective nucleotides were used with the EcoRI and MseI primer), (4) electrophoretic separation of the fragments in 6% denaturing polyacrylamide gels, and (5) silver staining for visualizing the fragments. The sequences of adaptors and primers are summarized in Table 1.

To detect the genomic differences between the male and female gibel carp, bulked segregant analysis (BSA) [23] for identification of sex-specific markers by AFLP was carried out as follows: female and male bulked samples were prepared from preamplification products. Nine female (D1♀) and nine male (D1♂) specimens were first used to do three bulks/each sex, and each bulk consisted of an equal amount of the preamplified DNA of three fishes. A total of 256 EcoRI/MseI primer combinations were screened in the six samples (female bulk 1, 2, 3 and male bulk 1, 2, 3). Subsequently, a primer combination (E15/M5) that produced a sex-specific fragment and the other six primer combinations (E3/M3, E6/M3, E6/M4, E9/M6, E9/M16, and E11/M13) with abundant AFLP bands in the bulk analysis were further analyzed in individual screening using 10 D1♀, 10 D1♂ fish, and their parents. Finally, the AFLP fragment found to be restricted to a single sex was cloned and sequenced as described [24].

2.4. Genome walking and sequence analysis

For further analysis, the Genome Walking Kit (Takara) was used to clone the flanking sequences of the putatively male-specific fragment. Briefly, according to the Kit instruction, three nested primers complementary to 3′ and 5′ ends of the fragment (SP1, SP2, and SP3, Table 2) were designed for thermal asymmetric interlaced PCR (Tail-PCR). Each of these primers was used in combination with four arbitrary degenerate primers available from Kit (AP1, 2, 3, 4), and thus, three rounds of nested amplification were made with the genomic DNA of a male gibel carp using eight different primer combinations in every round following the procedures described by the Kit instruction. PCR products were separated by 1% agarose gel electrophoresis. Then, the target fragments were extracted, cloned, and sequenced. Sequences were assembled and compared by DNAMAN software, and the homology of the sequences to known sequences was assessed by BLASTn and BLASTx in GenBank [25].

2.5. Conversion to male-specific SCAR marker (MSM)

Based on the flanking sequence of the male-specific AFLP fragment obtained by genome walking, a pair of locus-specific primers (*MSMF* and *MSMR*) was designed and used to amplify genomic DNA from 10 males and 10 females. The PCR was performed in a 25 μl aliquot of PCR mixture (20 ng of DNA, 50 nM of each dNTP, 5 pM of each primer, 2.5 μl 10× Taq buffer, 1.8 μl of $MgCl_2$ (25 mM), 1 U Taq polymerase (MBI)), and the final volume was made up with water. The PCR were carried out under the following conditions: initial incubation at 95 °C for 5 min, followed by 32 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, with a final extension of 10 min at 72 °C.

2.6. Testing genetic association of MSM to sex in gibel carp

The genetic association between the male-specific SCAR marker and sex was tested in 10 females (D1♀), 70 males (D1♂) from the male-biased mutant family, and 48 female (D2♀) and 31 male (D2♂) progeny from the cross between D1♀ and D1♂.

3. Results

3.1. Discovery of a male-biased mutant family in gynogenetic gibel carp

In contrast to all-females in gynogenetic gibel carp, a lot of males were unexpectedly observed from a clone D family produced by gynogenesis. In the propagation season of 2006, we individually detected the sex by the ovulated eggs or sperm. A total of 357 fishes were distinguished, and 347 individuals (97.2%) were identified as males (D1♂). Only 10 females (2.8%) (D1♀) were found in the family. Subsequently, we used them as parents to propagate their progenies (G2). The 2.8% females (D1♀) were demonstrated to produce gynogenetic all-female progeny when mated with a male common carp (C♂). Significantly, when the same female eggs were inseminated with the sperm from the mutant males, the reproduced progeny contained 22.1%

Table 2
Primers used in this study.

Application	Name	Sequence
Genome Walking	*M86Rsp1*	5′-AAACTGTACTAGATGAATGAGCCCG-3′
	M86Rsp2	5′-ACTAGATGAATGAGCCCGTTAGTGG-3′
	M86Rsp3	5′-GGTCTTCCGCTGCCACCTAGTTAA-3′
	M86Fsp1	5′-TTAACTAGGTGGCAGCGGAAGACC-3′
	M86Fsp2	5′-GAAGACCACTAACGGGCTCATTC-3′
	M86Fsp3	5′-CTAACGGGCTCATTCATCTAGTACAG-3′
SCAR Marker	*MSMF*	5′-ACTGCCATCTAACTCAGCCC-3′
	MSMR	5′-GCCACACTCACTTCTGTCTACA-3′

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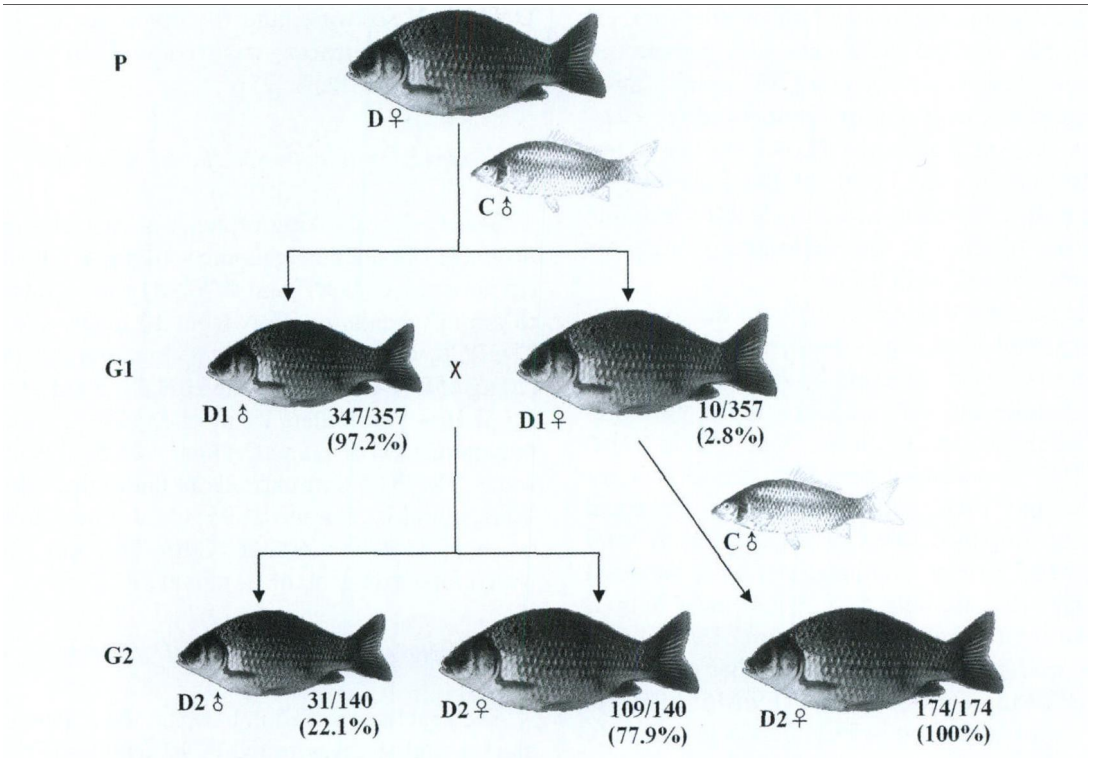

Fig. 1. Schematic diagram showing the discovery process of the male-biased mutant family in gynogenetic gibel carp.

males (31 males were found from 140 individuals). Fig. 1 summarizes the discovery process. The data indicate that we not only discover a male-biased mutant family in gynogenetic gibel carp, but also demonstrate that the male-determined genetic materials in the mutant males can transmit to the next generation.

3.2. Normal spermatogenesis in the males

Moreover, the spermatogenesis process in the mutant males was detected from the testicular sections stained with anti-CagVasa antibody immunofluorescence. As shown in Fig. 2, spermatogonia, spermatocytes, spermatid, and a large number of mature sperms are clearly distinguished in the testis, indicating that normal spermatogenesis has progressed in the mutant males.

3.3. Screening and isolation of a male-specific AFLP fragment

A total of 256 primer combinations were used to screen the AFLP differences between the male and female bulks, and only one primer combination E15/M5 was shown to produce a male-specific AFLP fragment, whereas others

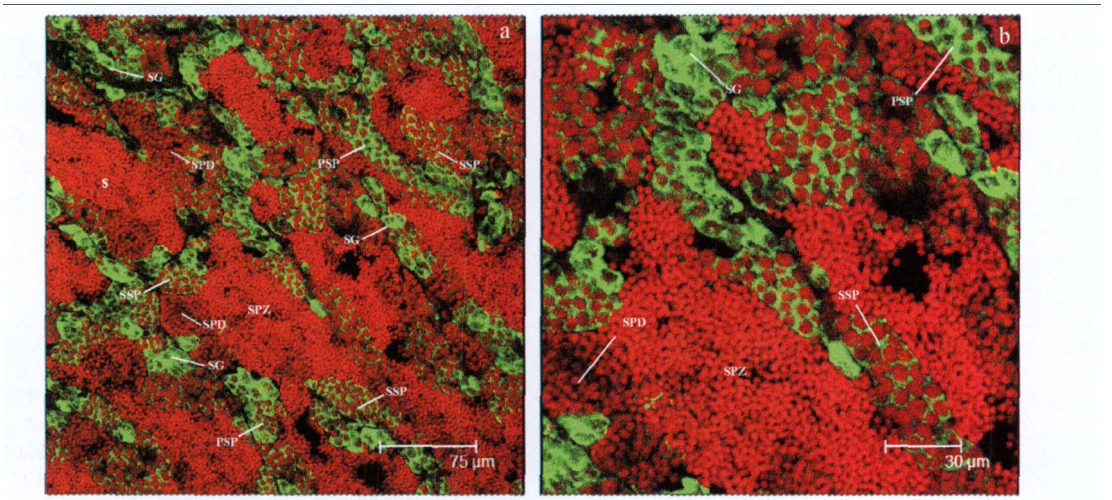

Fig. 2. Immunofluorescence detection of the male testicular section immunostained by the anti-CagVasa antibody. Red fluorescence stained by PI shows the nuclear position. SG, spermatogonia; PSP, primary spermatocytes; SSP, secondary spermatocytes; SPD, spermatids; SPZ, spermatozoa.

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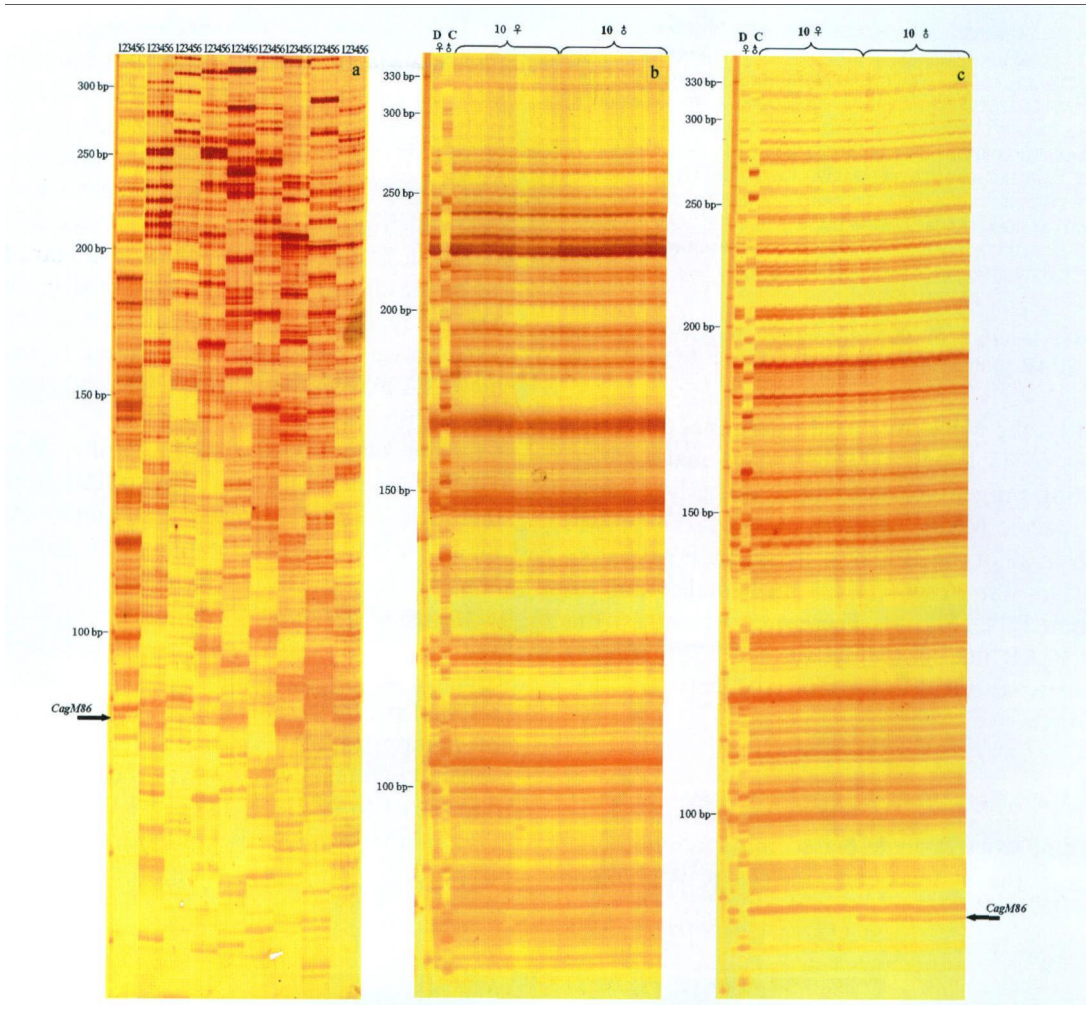

Fig. 3. Silver-stained AFLP amplification profiles between males and females in the male-biased mutant family. (a) Bulked segregant analysis of the male bulks and female bulks. Each primer combination includes six samples from left to right: 1-, 2-, and 3-male bulks; 4-, 5-, and 6-female bulks. (b) Individual screening of 10 females and 10 males. (c) Individual screening of 10 females and 10 males. The arrow indicates the male-specific AFLP fragment.

produced almost identical AFLP profiles between the male and female bulks (Fig. 3a). To confirm the identical genetic background, we further used six primer combinations to perform individual screening in 10 D1♀, 10 D1♂ specimens. As shown in Fig. 3b, identical AFLP profiles were detected in all males and females, and the profiles were the same as the maternal parent D♀. The identical genetic background verifies that all the males and females in the male-biased mutant family were indeed produced by gynogenesis from the maternal D♀.

Subsequently, the only male-specific AFLP fragment was further screened by individual screening using specimens of 10 females (D1♀), 10 males (D1♂), and their parents. As shown in Fig. 3c, the male-specific AFLP fragment was present in all male individuals and absent in all the 10 female individuals. Interestingly, the male-specific AFLP fragment was present in the maternal D♀, whereas no corresponding fragment was observed in the paternal red common carp (Fig. 3c). Moreover, the male-specific AFLP fragment was isolated, cloned, and sequenced. It contained 86 nucleotides, and was termed *CagM86*.

3.4. Conversion and confirmation of male-specific SCAR marker (MSM)

For further analysis and easy conversion to the SCAR marker, the flanking sequence of the male-specific AFLP fragment *CagM86* was cloned using the Genome Walking Kit (Takara). As a result, a total of 579 bp sequences were obtained from the male genome. Based on the 579 bp flanking sequences, a pair of locus-specific primers (*MSMF* and *MSMR*) was designed and used to detect the female and male specimens in the male-biased mutant family and the propagated progenies (Fig. 4).

First, a total of 70 males and 10 females were detected from the male-biased mutant family. As shown in Fig. 5, a male-specific fragment of about 500 bp was present in all specimens of the 70 males, whereas no band was observed in the 10 females. Once again, the male-specific fragment existed in the maternal gibel carp (D♀) but was absent in the paternal red common carp (C♂). The data further indicate that the male-specific 500 bp fragment is a good male-specific SCAR marker (MSM), and originates from the maternal parent (D♀).

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GAATTCATAAAAACTGTACTAGATGAATGAGCCCGTTAGTGGTCTTCCGCTGCCACCTA
MSMF→
GTTAAACTGCCATCTAACTCAGCCCGTGCCTCTCTCTCTGGGCCTCGCTGGTCTCCCTT
TCCGGAAAACCATACTGGATGTCTCCATCTGAAAAAAATAAAAAAAGATACATATGGGT
TAAGAGTCATGTAAATAGACTCTTAAACTTATAAGAGTGTAAGGGAAGATGAAAGAGT
GCTTAAATTCTAAAAATACACTTTTAAGTGCTTGATGAAGACACAGAAAGCTGTCGAG
ATTTTAAACTATATACAAGTACACAAGCCTTTTATATAAAATATTTTGCTTTGTAACAAAA
TAAGAAAAACCTGTTTTAACTTAAAAATTTGCTTTAAATGTAAAGTTTAAGTTTAAATTA
TATTAATTGTTGAATCATAAAATTAACACAAAGAACTCATTATGAATGAGAATGAATAAA
CTTGGGATATATATCTTTATTTTACAGTCACACAATAAAACACAATATACATATGTATAAA
ATAAGAGCAGGTCCATTCCACATGTAGACAGAAGTGAGTGTGGC
←*MSMR*

Fig. 4. Nucleotide sequence of the male-specific fragment *CagM86*. Positions of the SCAR primers are underlined.

Subsequently, the MSM was used to examine the genomic specimens of 48 females (D2♀) and 31 males (D2♂) from the mating progeny between D1♀ and D1♂. Consequently, the specific MSM fragment of about 500 bp was amplified only in all of the 31 males and the paternal parent D1♂, whereas it was absent in all the females and the maternal parent D1♀ (Fig. 6). Therefore, we suggest this male-specific SCAR marker (MSM) should be an effective marker in genetic sex identification of gibel carp.

4. Discussion

In the current work, a male-biased mutant family was discovered from the gynogenetic gibel carp. And based on the discovery, a male-specific SCAR marker was isolated and identified from this male-biased mutant family. The successful isolation and identification mainly depended on the gynogenetic background, because the nearly identical AFLP profiles between the males and females provide a great benefit to screen and distinguish the male-specific fragment. Significantly, the male-specific SCAR marker is present in males of the male-biased mutant family, and it can be steadily transmitted to the next generation. Moreover, the male-specific DNA fragment is strictly associated to the progeny males. So far, it should be the first sex-specific DNA marker identified from the gibel carp and from a triploid fish species.

In the male-biased mutant family, the male-specific SCAR marker was typically amplified from genomes of all the 97.2% male progeny but absent in all the 2.8% females, whereas in the parents, the male-specific DNA sequence was surprisingly found to exist in the genome of the maternal parent (D♀) but absent in the paternal common carp (C♂), indicating that the male-specific DNA sequence in the male progeny was inherited from the maternal parent through eggs. Why did the maternal parent contain the male-specific sequence but develop to be a phenotypic female? How was the male-specific sequence transmitted to 97.2% of the eggs? Why did it disappear in 2.8% of the eggs? These questions are very interesting, but hard to clarify. According to the obtained data, there

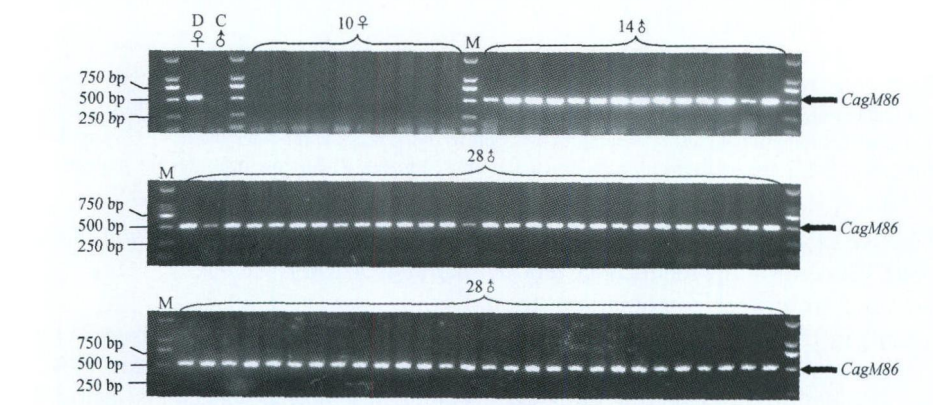

Fig. 5. PCR detection of the male-specific SCAR marker (MSM) in 10 females and 70 males from the male-biased mutant family plus their parents. The amplified male-specific fragment is indicated by an arrow.

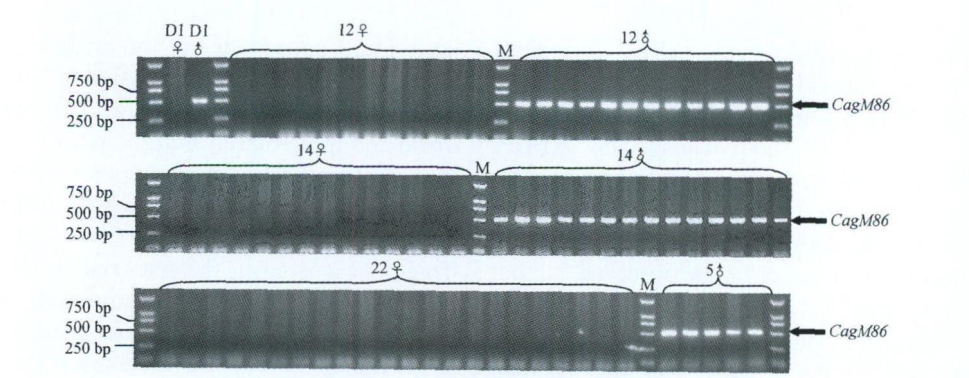

Fig. 6. PCR detection of the MSM in the mating progeny between D1♀ and D1♂. The amplified male-specific fragment is indicated by an arrow.

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are two possible explanations. First, the maternal parent (D♀) might have obtained the genetically male-determined factors through a particular pattern, such as by incorporation of subgenomic amounts of DNA [6]. Then, after being transmitted to most of its progeny through eggs, the male-determined factors may be steadily fixed and reactivated, and the eggs will develop into males. And the 2.8% of females might have resulted from a few eggs that did not inherit the male-determined factors, because the supernumerary chromosomes may distribute unequally to the gametes [26,27]. For the other possibility, as described in many fish species [28–30], the sex of the gibel carp may also be flexible and can be affected by the environment and some unknown factors, and thereby, the maternal parent (D♀) may be a physiological female with a genetically male genome, but sex reversal might have been induced by some unknown factors. Hence, in order to unveil the actual explanation, we can use the male-specific SCAR marker to search more phenotypic females, which contain this marker, from the cultural and natural population. Additionally, artificial sex reversal of gibel carp by physical or chemical factors, such as high temperature, hypoxia and estrogen, should be tried in further work, and then, the male-specific SCAR marker can be used to accurately identify the artificial physiological females. Possibly, artificial male-biased gynogenetic families may be reproduced by these physiological females, and genetically male-biased embryos and individuals may be produced easily for the purpose of research on sexual development in gibel carp.

In mammals, the sex determining factor, SRY (sex-determining region-Y chromosome), has been identified in most of the species including marsupials [31–33]. For non-mammals, no widely conserved sex-specific genes have yet been described. In 2002, the first sex-determining gene of the non-mammalian vertebrate dmY/dmrt1Y was identified in medaka [34], but it is not the universal primary sex-determining gene in fishes [35]. Sex-determination systems among fish species are considerably more variable and subject to exogenous influence than in mammalian sex determination [36]. Sex determination in different fish species can be regulated by environmental factors, or by genetic systems that may include poly-factor or single-factor (XY or ZW) systems [37,38]. However, little is known about the mechanisms of sex determination in the triploid fish species. The identification of the male-specific marker of gibel carp should be the first step in studying the sex determination system in the triploid fish species. Based on this male-specific DNA sequence, the chromosomal location of the male-specific fragment in gibel carp can be studied by fluorescence *in situ* hybridization and other molecular cytogenetic methods, and the transmittal pattern of the male-determination factors during oogenesis and spermatogenesis in the triploid gibel carp will be investigated in further work. Genomic walking, screening of the BAC library [17] and other methods can be used to search more genetic factors linked to this male-specific DNA sequence, and then to research the primary sex-determining locus and ultimately to isolate the major genes in the gynogenetic gibel carp. Moreover, research will help us to explore the relationship between the male-specific SCAR marker and male formation in the triploid gynogenetic species, and will be of great significance in the sexual evolution of vertebrates.

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