J Mol Evol (2000) 51:498–506
DOI: 10.1007/s002390010113

JOURNAL OF MOLECULAR EVOLUTION

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Genetic Evidence for Gonochoristic Reproduction in Gynogenetic Silver Crucian Carp (*Carassius auratus gibelio* Bloch) as Revealed by RAPD Assays

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Received: 5 January 2000 / Accepted: 3 August 2000

Abstract. Sex evolution has been a debating focus in evolutionary genetics. In lower vertebrates of reptiles, amphibians, and fish, a species or a bioform reproduces either sexually or asexually but never both. A few species were found to consist of all females in fish. These all-female species can propagate by asexual reproduction modes, such as gynogenesis and hybridogenesis. However, the coexistence of sexuality and asexuality in a single species was recently noted only in a cyprinid fish silver crucian carp, *Carassius auratus gibelio.* This fish had been demonstrated to be capable of gynogenesis stimulated by sperm from other related species. Surprisingly, natural populations of this fish consist of a minor but significant portion (approx. 20%) of males. As different clones with specific phenotypic and genetic characteristics have been found, and RAPD markers specific to each clone have recently been identified, this fish offers many advantages for analyzing whether or not genetic recombination occurs between different clones. In this study, artificial propagation was performed in clone F and clone D. Ovulated eggs from clone F were divided into two parts and respectively inseminated with sperm from a clone D male and from a red common carp (*Cyprinus carpio*) male. The control clone D individuals were selected from gynogenetic offspring of clone D activated by sperm of red common carp. The phenotype and sex ratio in the experimental groups were also observed. Using RAPD molecular markers, which allow for reliable discrimination and genetic analysis of different clones, we have revealed direct molecular evidence for gonochoristic reproduction in the gynogenetic silver crucian carp and confirmed a previous hypothesis that the silver crucian carp might reproduce both gynogenetically and gonochoristically. Therefore, we conclude that the silver crucian carp possesses two reproductive modes, i.e., gynogenetic and gonochoristic reproduction. The response mechanism of two reproductive development modes may be the first discovery in vertebrates. Additionally, we discuss the evolutionary implication between gynogenetic and gonochoristic reproduction modes and the contribution of the minor proportion of males to genetic flexibility in the gynogenetic silver crucian carp.

Key words: Silver crucian carp — Gynogenesis — Gonochoristic reproduction — Sex evolution — Genetic recombination — RAPD markers

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Introduction

There are two major forms of reproduction in the animal kingdom: sexuality and asexuality. In sexuality meiosis gives rise to two types of haploid gametes: sperm and eggs in the male and female gonads, respectively. Fusion of an egg and a sperm during syngamy leads to the formation of a diploid zygote, which develops into a male or female adult of the next generation. Genetic crossing-over and recombination during meiosis and syngamy ensure tremendous diversity or genetic variation. Asexuality is adopted by all-female populations

where meiosis and syngamy are absent (Maynard Smith 1998).

Asexuality is rare in vertebrates. Since the first gynogenetic fish, the Amazon molly *Poecilia formosa,* was found by Hubbs and Hubbs (1932), about 50 unisexual species that are composed of all-female individuals have been reported in lower vertebrates (Vrijenhoek et al. 1989). These unisexual species were identified to reproduce by gynogenesis, hybridogenesis, or parthenogenesis (Gui 1989) and have long been a central paradigm in evolutionary genetics (Crow and Kimura 1965; Kondrashov 1994; Schartl et al. 1995; Joshi and Moody 1998). Owing to the absence of genetic recombination and the accumulation of deleterious mutations, unisexual species were generally thought to be short-lived on an evolutionary time scale because of Muller's ratchet (Muller 1964; Crow 1999). Actually, comparative studies on mitochondrial genome sequences in gynogenetic salamanders (Hedges et al. 1992; Spolsky et al. 1992) and in hybridogenetic *Poeciliopsis* fish (Quattro et al. 1992) suggested that unisexual species were genuinely old in evolutionary terms. However, how do the species evolve and manage the long longevity and history without recombination? The intriguing contradiction between the deleterious mutation accumulation and the evolutionary longevity seems more prominent, and the underlying mechanism remains to be revealed and interpreted (Maynard Smith 1992). A gynogenetic silver crucian carp (*Carassius auratus gibelio*) discovered in China (Gui 1996, 1997) provides a good model system for pursuing the evolutionary problem, because a certain proportion of males has been discovered in the gynogenetic crucian carp (Jiang et al. 1983).

The gynogenetic crucian carp was originally described in a population from the Shuangfeng Reservoir in northern China and was identified as a triploid fish (Shan and Jiang 1988). Gynogenesis has been demonstrated using heterologous sperm from other fish species to activate egg and embryo development (Yu 1982; Jiang et al. 1983). And surprisingly, the heterologous sperm, besides its role in activating the development of an egg, is able to contribute to the phenotype of the gynogenetic offspring. This novel gynogenesis has been referred to allogynogenesis (Jiang et al. 1983). In comparison with the relative gynogenetic crucian carp reported in Russia (Cherfas 1981) and in Japan (Kobayasi 1971), as well as other gynogenetic species in vertebrates (Vrijenhoek 1994), the gynogenetic fish is more interesting. It exists as a bisexual population consisting of females as the majority and males as the minority (approximately 20%) in natural habitats (Jiang et al. 1983; Gui 1997), whereas other gynogenetic species are composed of all-female populations (Vrijenhoek 1994). Preliminary experiments showed that the survival offspring could be reproduced by inseminating eggs of the females with sperm from the males, and male individuals occurred in the offspring (Jiang et al. 1983). These special features lend the unique silver crucian carp as an intermediate evolutionary system of vertebrates between uni- and bisexual reproduction and as a more unique model to understand the evolutionary genetics of gynogenesis and to elucidate the regulatory mechanism underlying the reproduction mode in vertebrates.

Gynogenetic crucian carp were found to consist of various clones (Zhu 1990). More than six clones, including natural and artificial clones, have been obtained and maintained at the Guanqiao Experimental Station at our Institute (Fan et al. 1997; Zhou et al. 2000). There exist differences among these clones in body type, growth, spawning time, chromosome number, serum protein phenotypes, and other characters (Zhu and Jiang 1987, 1993). RAPD molecular markers have been detected recently by us in different gynogenetic clones of the silver crucian carp (Zhou et al. 1998, 2000) and provide a valuable and powerful tool for analyzing whether or not genetic recombination occurs between different clones. In this study, we reveal direct molecular evidence for gonochoristic reproduction in the gynogenetic silver crucian carp and discuss the evolutionary implication between gynogenetic and gonochoristic reproduction modes.

Materials and Methods

Source of Samples. Clone F was used as the maternal donor in artificial propagation experiments, because it shows obvious phenotype characteristics, with a bulge on the anterior back just behind the head (Fan et al. 1997; Zhou et al. 2000). The mated males between clones were selected from clone D. Control gynogenetic individuals of clone F were inseminated by sperm from red common carp (*Cyprinus carpio*) to activate the eggs. Generally, spawning was artificially induced by two intraperitoneal injections with a mixture of acetone-dried carp pituitary, hCG and LRH-A (Gui 1999). Ovulated eggs from clone F were divided into two parts and respectively inseminated with sperm from a clone D male and from a red common carp male. The offspring produced were cultured in separate fishponds and called FD and F, respectively. The control clone D individuals were selected from gynogenetic offspring of clone D activated by sperm of red common carp.

DNA Preparation and RAPD-PCR Reactions. Total genomic DNA was isolated from the liver, referring to the standard phenol–chloroform extraction procedures (Sambrook et al. 1989). Because previous studies demonstrated that most intraclone individuals produced identical RAPD band patterns, i.e., there is a high genetic homogeneity for each clone (Zhou et al. 2000), only three and five adult individuals were sampled from gynogenetic clone F and clone D. In preliminary RAPD fingerprint screening, six individuals, including three females and three males, were sampled from FD offspring. RAPD amplification reactions were performed as described previously (Zhou et al. 1998, 2000).

RAPD Data Analysis. Thirty-three primers were found to produce well-amplified and reproducible electrophoretic bands of the applied 40 primers. Using these informative band patterns produced by the 33 primers, all individual, a total of 2268 distinguishable fragments (162 per individual), including universal and specific, was scored, with data coded as a vector of 1 or 0, representing band presence or absence,

respectively. Pairwise distance matrices among these individuals of three clones were estimated based on Apostol (1993), and a phylogenetic tree showing the hierarchical structure of RAPD pattern affinities among individuals and clones was constructed from the distance matrices by NJTREE analysis, as implemented in the program RAPDistance 1.04 (Apostol 1993).

Purification of Specific DNA Fragments. After running products of RAPD reactions in agarose gel, the selected DNA fragments were excised and put into 1.5-ml tubes. The DNA fragments were purified using a Biostar Glassmilk DNA Purification Kit (BioStar International, Canada). Briefly, 3 vol of NaI was added to the tubes, which were then incubated at 55°C to melt the gel. Glassmilk was suspended well and 5 μl was added to the tubes. On mixing and incubating at room temperature for 5 min, DNA was bound to glassmilk. The glassmilk/DNA complex was pelleted by spinning for 5 s at full speed. The supernatant was discarded and the glassmilk/DNA was resuspended in 0.5 ml of cold wash buffer. Spinning and washing of the pellet were repeated an additional two times. The supernatant was discarded and the pellet was dried at room temperature for about 10 min. Deionized water (10 μl) was added, and the pellet was resuspended well and centrifuged for 30 s. The supernatant containing the eluted DNA was removed carefully and used directly for ligation.

Cloning of Specific DNA Fragments. Ligation reactions were performed in a 10-μl volume containing approximately 50 ng pure DNA, 50 ng pGEM-T Easy Vector (Promega), and 1 μl T4 DNA ligase (3 Weiss U/μl) and incubated overnight at 4°C. Ligation reactions were transferred into tubes containing OH52 High Efficiency Competent Cells. The tubes were flicked gently to mix and placed on ice for 20 min. After heat shocking the cells for 40–50 s in a water bath at exactly 42°C, the tubes were returned to ice for 2 min. LB medium (1 ml) was added to the tubes, which were then incubated for 1.5 h at 37°C with shaking (150 rpm). The cells were pelleted by centrifugation, resuspended in 100 μl LB medium, and plated on 2 LB/ampicillin/IPTG/X-Gal plates. Then the plates were incubated overnight at 37°C. White colonies were selected, put in tubes containing 4.5 ml LB and ampicillin, and incubated overnight at 37°C. The clones were screened by PCR. PCR reactions were performed in a 20-μl volume containing 1 μl of culture as template DNA, a 0.2 μ*M* concentration of each primer (M13+ and M13–), 0.5 U of Taq DNA polymerase (BioStar International, Canada), a 0.1 μ*M* concentration of each dNTP, and 1× buffer for Taq DNA polymerase (BioStar International, Canada). Amplification was also performed in a Perkin–Elmer DNA GeneAmp PCR System 9600, with 50°C as the annealing temperature.

Purification and Sequence Analysis of Cloned Plasmid DNA. Pure plasmid DNA was obtained using Min-M Plasmid Miniprep (Viogene) according to the manufacturer's protocol. Briefly, 5-ml overnight cultures were spun at 4000*g* for 5 min to pellet cells. After adding 250 μl of solution I (suspension), solution II (lysis), and solution III (neutralization), the tubes were spun at 10,000*g* for 5 min. The supernatants were put in a column and spun at 10,000*g* for 5 s. Wash I buffer (0.5 ml) was added, and the mixture spun at 10,000*g* for 30 s. Then 0.7 ml of wash II buffer was added, and the mixture spun at 10,000*g* for 30 s. Water (50 μl) was added to the column, allowed to stand for 30 s, and spun at 10,000*g* for 1 min. The eluted DNA can be used to sequence directly. DNA sequencing of specific DNA fragments was performed by the chain termination reaction with a dRhodamine terminator cycle sequencing kit (PE; Catalog No. 403042) and an ABI PRISM 310 Genetic Analyzer (Perkin Elmer) according to the provided protocol. Reactions were performed in a 20-μl volume containing 8.0 μl of Terminator Ready Reaction Mix, 400 ng of template DNA, and a 3.2 p*M* concentration of M13+ or M13– primer. Amplifications were performed using a Perkin–Elmer DNA GeneAmp PCR System 9600. The amplification program was 25 cycles of 10 s at 96°C, 5 s at 50°C, and 4 min at 60°C. After amplification, the contents of each reaction were pipetted into a 1.5-ml microcentrifuge tube. To each tube was added 74 μl of 70% EtOH/0.5 m*M* $MgCl_2$, followed by brief vortexing. The tubes were left at room temperature for 15 min to precipitate the products and spun for 30 min at maximum speed. The supernatants were aspirated carefully. After drying, the sample pellets were resuspended in 12 μl of template suppression reagent, vortexed, and spun. The samples were heated at 95°C for 2 min to denature, chilled on ice, vortexed and spun again, then placed on ice until ready to use. Electrophoresis was performed on the ABI PRISM 310 Genetic Analyzer.

PCR Detection of Specific DNA Fragments. A pair of primers (CCTCTCGACAGCAGTAATA and CCTCTCGACACTCCCTTAC) was synthesized (Sangon, Shanghai) according to the obtained nucleotide sequences and the principle of sequence characterized amplified regions (SCARs) and was used to identify the specific DNA fragments produced by Opj-7 in the offspring of clone F × clone D and in clone D by PCR reactions similar to those described above. The annealing temperature was 60°C.

Results

Variations of Survival Rate, Phenotype, and Sex Ratio in Mated FD Offspring Between Clones

Artificial propagation experiments indicated that when clone F eggs were inseminated by sperm from a clone D male, they could also reproduce offspring, but the survival rates of FD offspring were lower than that of the control gynogenetic clone D at the hatching and first-feeding larvae stages (Fig. 1). In contrast to the identical phenotype in the gynogenetic offspring of clone F stimulated by heterologous sperm of red common carp, the adults in mated FD offspring differentiated into three phenotypes. One phenotype is similar to that of clone F, and the second is similar to clone D. The third varied differently from clone F and clone D, showing a longer and thinner body type (Fig. 2).

Sex differences were significant between gynogenetic clone F and the mated FD offspring. No males were found in the control clone F offspring that resulted from gynogenesis by red common carp sperm. Males were detected in the FD offspring, and the percentage of males was 14.1% (39 males of 276 individuals). The differences implied that silver crucian carp eggs might possess different response mechanisms to different sperm, and the sperm from clone D might make a genetic contribution to its offspring.

Dendrogram Relationship of RAPD Fingerprints Among Mated FD Offspring, Gynogenetic Clone F, and Gynogenetic Clone D

RAPD fingerprints have been confirmed to be effective and powerful markers to discriminate different clones, because the individuals from each clone display homogeneity in RAPD patterns, while different clones exhibit various characteristic RAPD patterns (Zhou et al. 2000). Six individuals from the mated FD offspring, three from

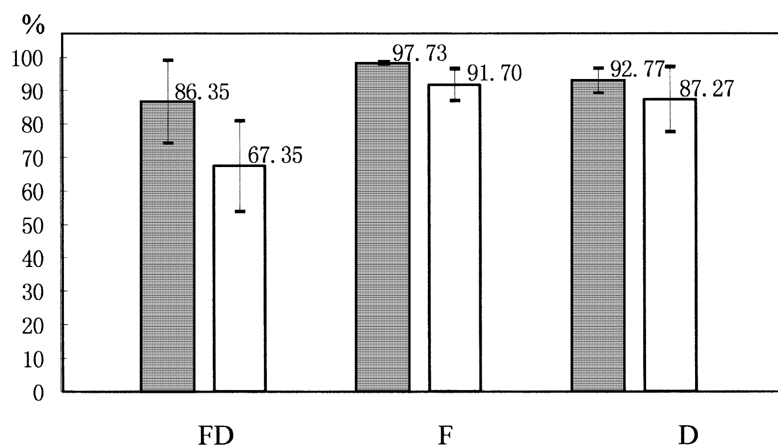

Fig. 1. Hatching rates (*shaded*) of embryos and survival rates (*open*) of first-feeding larvae in three experimental groups of silver crucian carp. The eggs of clone F were respectively inseminated with sperm from clone D (FD) and from red common carp (F), and the eggs of clone D were inseminated with sperm from red common carp (D).

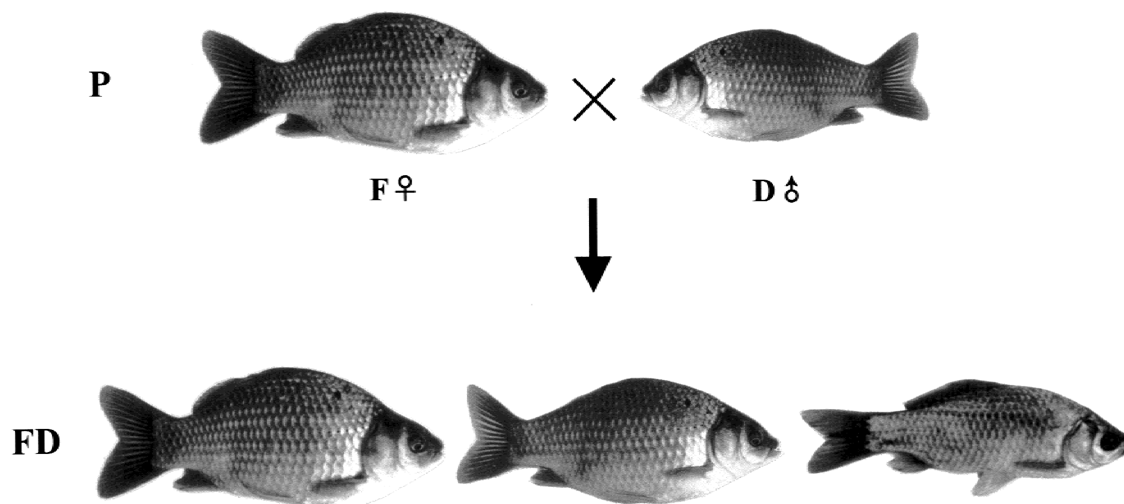

Fig. 2. Phenotypes of parents (F and D) and offspring (FD) used in the study. The offspring differentiated into three phenotypes. One phenotype is similar to that of clone F, and the second is similar to that of clone D. The third varied differently from those of clone F and clone D, showing a longer and thinner body type.

gynogenetic clone F, and five from gynogenetic clone D were screened using 40 10-nucleotide-long random primers. Of these primers, 33 produced well-amplified and reproducible band patterns (1 to 12 bands ranging in size from about 0.1 to 3.0 kb). After excluding bands that were not clearly identifiable, a total of 2268 distinguishable fragments (162 per individual) was compiled for phylogenetic relationship analysis. The average genetic distances among the 14 individuals were obtained with the RAPDistance 1.04 program. The data (Table 1) indicated that the average genetic distances among individuals of gynogenetic clone F and clone D are much lower (about 0.01) than those (about 0.04) among individuals in FD offspring. Compared with the average genetic distances between F and D (0.35), the average genetic distance between FD and D is larger (about 0.34) than that between FD and F (0.30). The dendrogram (Fig. 3) among 14 individuals of the mated FD offspring, clone F, and clone D clearly indicated their divergence and similarity relationships. FD was clustered together with clone F, showing more relation to clone F than to clone D. We can consider that FD is a new clone that is not same as clone F or D. But how was FD produced? Obviously, the small subgenomic amounts from the paternal genome and mutations cannot explain this. The average genetic distance among the individuals of clone D is only about 0.01, while the genetic distances between FD and clone F are about 0.30.

Differentiation Between Females and Males in Mated FD Offspring

Compared with gynogenetic clones F and D, there are more variations among individuals in FD offspring, and these variations appear mainly in male individuals (Fig. 4). The estimated average genetic distances (Table 2) indicated that the distances (about 0.033) among female individuals are smaller than those (about 0.047) among male individuals. This differentiation phenomenon between males and females implied that the production of males might be associated with genetic recombination.

Table 1. Average genetic distances among three experimental groups of silver crucian carp

	FD	F	D
FD	0.0439 ± 0.0140		
F	0.3076 ± 0.0144	0.0145 ± 0.0156	
D	0.3405 ± 0.1221	0.3532 ± 0.0074	0.0124 ± 0.0051

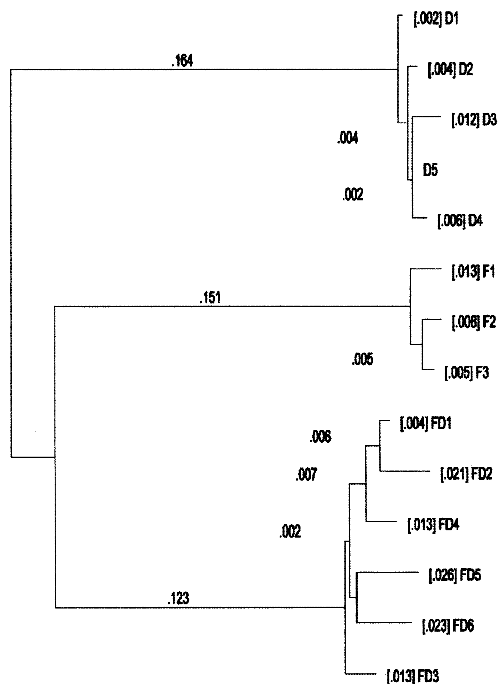

Fig. 3. Dendrogram of the 14 individuals analyzed among three experimental groups of gynogenetic silver crucian carp obtained by NJTREE analysis in the RAPDistance program.

RAPD Marker Evidence for Genetic Recombination in Mated FD Offspring

There are identical RAPD fingerprints among individuals in gynogenetic clone F or clone D, which is consistent with previous results (Zhou et al. 2000). In contrast with gynogenetic clone F, the mated FD offspring amplified different fingerprints than clone F. More interestingly, most of these different DNA fragments obviously originated from clone D. For example, primers of Opj-7 and Opq-8 produced specific fragments shared by FD and clone D (Fig. 5). Obviously, these shared fragments should come from clone D. In some RAPD patterns, FD offspring not only produced shared fragments with clone D, but also lacked specific bands for clone F (Fig. 6). This implied that the FD individuals both received some genetic materials from clone D and lost some genetic materials that belong to clone F. Some primers, such as Opj-1, amplified novel fragments in the FD offspring (Fig. 6). This direct marker evidence suggests that genome recombination between clone F and clone D should have occurred in the mated FD offspring.

Sequence Analysis and PCR Amplification of Shared Fragments Between FD Offspring and Clone D

One shared DNA fragment among individuals in the mated FD offspring and in clone D revealed by primer Opj-7 was cloned from the respective amplified product. Sequencing data indicated that both of the cloned DNA fragments were of identical nucleotide sequences, having only subtle differences (Fig. 7A). According to the nucleotide sequences, we designed and synthesized a pair of PCR primers to identify the specific fragments. As expected, the vast majority of FD individuals (17 of 18 individuals analyzed) and the individuals in clone D amplified the specific DNA fragment, while no products were detected in clone F (Fig. 7B). Moreover, in three kinds of individuals with different phenotypes differentiated from the mated FD offspring, the specific DNA fragments shared with clone D were also demonstrated to exist (Fig. 6). These data confirmed that the specific DNA fragments should be homologous between the FD offspring and clone D, and the fragments in the FD offspring should originate from the mated male of clone D. It was suggested that sperm–egg fertilization and genome recombination might have occurred in the insemination of clone F with clone D.

Discussion

Asexual reproduction has been a debating focus of sex evolution (Barton and Charlesworth 1998). Higher vertebrates, mammals, and birds, reproduce exclusively sexually. In lower vertebrates, reptiles, amphibians, and fish, a species or a bioform reproduces either sexually or asexually but never both. A few species of fish, including the Amazon molly *Poecilia formosa* (Hubbs and Hubbs 1932), *Poeciliopsis 2monacha-lucida, Poeciliopsis monacha-2lucida, Poeciliopsis monacha-lucida-ocicdentais* (Cimino 1972), *Carassius auratus gibelio* (Cherfas 1981; Jiang et al. 1983), *Carassius auratus langsdofii* (Kobayasi et al. 1977), *Menidia clarkhubbsi* (Anthony and Mosier 1982), and *Phoxinus eosneogaeus* (Goddard et al. 1998), were found to consist of all females. These all-female populations can propagate by asexual reproduction modes, such as gynogenesis and hybridogenesis (Gui 1989; Schlupp et al. 1998). However, the coexistence of sexuality and asexuality in a single species has recently been noted only in the cyprinid fish silver crucian carp, *Carassius auratus gibelio.* This fish usually adopts gynogenesis. Surprisingly, natural populations of this fish consist of a minor but significant portion (approx. 20%) of males that appear to be identical to females in DNA content and karyotype (Fan and Shen 1990). In a previous study, the RAPD technique was used for analyzing clonal heterogeneity in the unique silver crucian carp, and five gynogenetic clones with specific RAPD markers were revealed (Zhou et al.

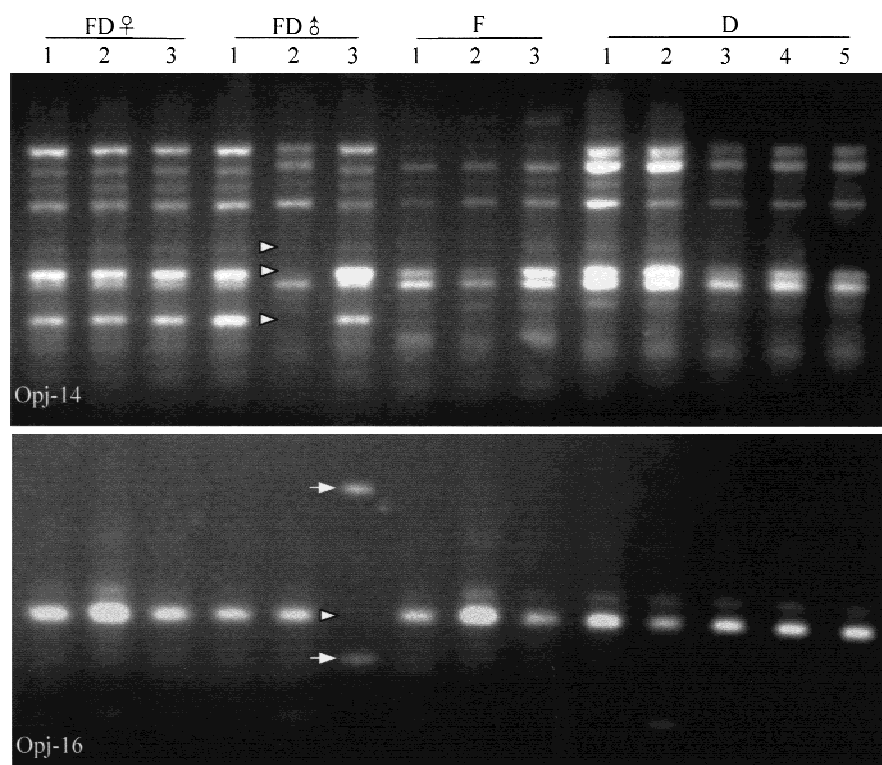

Fig. 4. Electrophoretogram of two typical RAPD products obtained from the Opj-14 and Opj-16 primers. The *arrows* indicate the various bands appearing in male individuals of FD offspring.

Table 2. Average genetic distances between female and male individuals in the FD offspring

	FD (♀)	FD (♂)
FD (♀)	0.0331 ± 0.0102	
FD (♂)	0.0456 ± 0.0072	0.0469 ± 0.0159
F (♀)	0.2974 ± 0.0125	0.3199 ± 0.0105
D (♀)	0.3383 ± 0.1444	0.3424 ± 0.0296

2000). In the current study, we have further revealed direct molecular evidence for gonochoristic reproduction in the gynogenetic silver crucian carp by the powerful RAPD tool.

As shown in the above results, the mated FD offspring amplified different fingerprints from clone F, of which some were shared by the FD and clone D (Figs. 5 and 6) and some were lacking in the FD and clone D (Fig. 6). How are these DNA fragments produced or not produced? Obviously, these fragments implied that the FD individuals both received some genetic materials from clone D and lost some genetic materials that belong to clone F. This direct marker evidence suggests that genome recombination between clone F and clone D should have occurred in the mated FD offspring. Average genetic distances (Table 1) and phylogenetic dendrograms (Fig. 3) within and among the three analyzed experimental groups also indicated their divergence and similarity relationships due to genetic recombination between the two clones.

An amazing question in the gynogenetic silver crucian carp is the origin of males. Artificial propagation experiments showed that male individuals occurred in the offspring reproduced by inseminating eggs of the females with sperm from the males (Jiang et al. 1983), but the reason why and how they are produced is obscure. Because genetic recombination in the mated offspring between two clones had been revealed by the RAPD assays, the current study provided direct genetic evidence for male occurrence, i.e., males might be produced by a gonochoristic reproduction mode similar to bisexual reproduction. Additionally, there are more variations among individuals in FD males than in FD females (Fig. 4). Estimated average genetic distances (Table 2) also indicated that the distances among female individuals are smaller than among male individuals. This differentiation phenomenon between males and females further suggested that the production of males should result from genetic recombination between the two clones.

Another, more amazing question is why there is a bias toward a sex ratio in the offspring reproduced by the gonochoristic mode. Why is the sex ratio not 1:1? The first might be related to high numbers of chromosomes. Many studies indicated that chromosomal polymorphism existed in the silver crucian carp (Zhu and Jiang 1993) and was characterized by triploidy and tetraploidy (Gui et al. 1993a). As demonstrated previously, most unisexual bioforms in vertebrates are frequently associated with genome polyploidy (Gui 1989; Vrijenhock et al. 1989; Kondrashov 1994). The higher ploidy may be one of the reasons for the bias toward a sex ratio. The second might be correlated with genetic imprinting between sexes. Imprinting can permit one parent to stifle the ge-

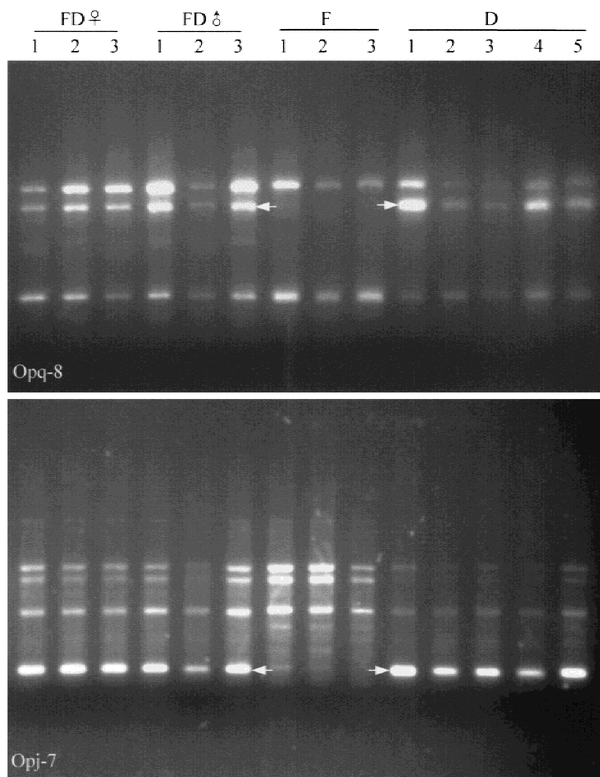

Fig. 5. Electrophoretogram of two typical RAPD products obtained from the Opq-8 and Opj-7 primers. The *arrows* show specific fragments shared by clone D and the mated FD offspring.

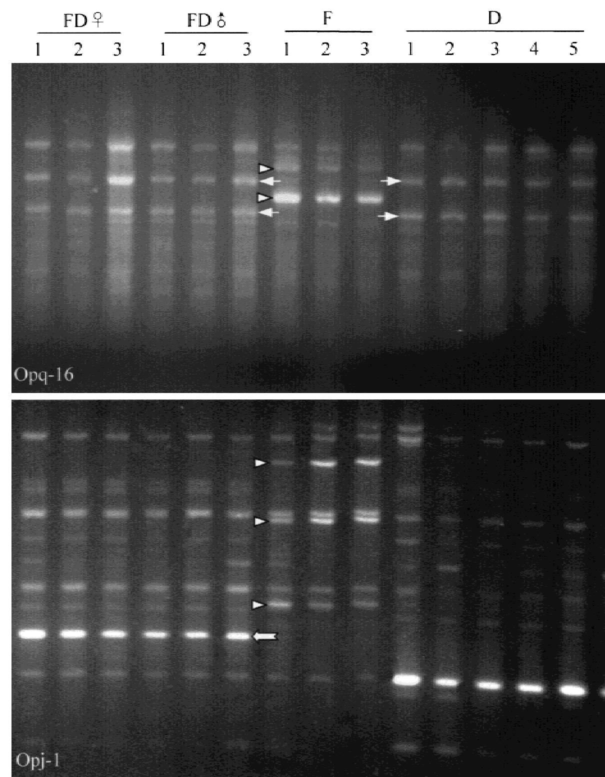

Fig. 6. Electrophoretogram of two typical RAPD products obtained from the Opq-16 and Opj-1 primers. The *arrows* show specific fragments shared by clone D and the mated FD offspring. The *triangles* show the fragments lacking in the mated FD offspring. The *arrowhead* shows a novel fragment produced in the mated FD offspring.

netic contributions of the other. In some insects, males silence and then discard entire sets of chromosomes, while in plants and mammals the effects of imprinting are more subtle, as often individual genes rather than whole chromosomes are marked (Pennisi 1998). In the "maternal society" of silver crucian carp, the female achieves absolute superiority over the male in the struggle between the maternal and the paternal genes. In the process of fusion of the female pronucleus and male pronucleus, the maternally derived genes can selectively mark, then silence the paternal copies of genes, especially the genes specific to males, or even discard the male chromosomes, in ways that advance that female's genetic interest. Only a few genes specific to males can escape, not to be marked and impressed. So only a few males (5–25%) are produced.

Cytological investigation confirmed that there was no extrusion of the first polar body during oocyte maturation in the silver crucian carp (Ding and Jiang 1991). And spindle behavior changes were significantly different from those in a related subspecies which has dimorphic sex. The chromosomes were first arranged to become a tripolar spindle, then the tripolar spindle was turned around and reunited mutually, and, finally, a normal bipolar spindle was formed (Yang et al. 1999). In combination with the current RAPD molecule evidence, the previous hypothesis that the silver crucian carp might reproduce both gynogenetically and gonochoristically (Gui et al. 1993b) was confirmed. The two reproductive modes were first revealed in multiple tetraploid crucian carp with 212 chromosomes, of which 162 originated from clone D of the silver crucian carp and 50 from the haploid sperm of red common carp (Gui et al. 1993a, b). Therefore, we can conclude that the silver crucian carp possesses two reproductive modes, i.e., gynogenetic and gonochoristic reproduction. When the eggs are inseminated with heterologous sperm from other species, the entered sperm does not decondense until the first cleavage and trigger embryogenesis. In this case, the paternal genome makes no contribution or incorporates only subgenomic DNA amounts. This responding reproduction mode leads to a clonal lineage of genetically identical individuals, with no males found. It is a typical gynogenesis. However, when the eggs are inseminated with homologous sperm from silver crucian carp males, the entered sperm decondenses and becomes a male pronucleus, and then the male pronucleus undergoes fusion with the female pronucleus. The fused nucleus of the zygote undergoes recombination and removes extra chromosomes (about half of the maternal chromosomes) from the egg as another polar body or holds in the egg until disintegration. In this case, the responding mode produces genetically diverse offspring, and males are produced in the progeny. This is similar to gonochoristic

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a   CCTCTCGACAGCAGTAATACAGGATTATTTGTGACCCTGGAGCACAAAAGCAGTTTTAAGTCGCTGGGGTATATTTGTA
b   CCTCTCGACAGCAGTAATACAGGATTATTTGTGACCCCGGAGCACAAAAGCAGTTTTAAGTCGCTGGGGTATATTTGTG

    GCAATAGCCAAAAAAAAAAACGT    ATCAGTCAAAATAATACATTTTTATTTTATGCTAAAAATCAGTAGGATATTAA
    GCAATAGCCAAAAAAAAAACCGTTGTATCAGTCAAAATAATAAATTTGTATTTTATGCTAAAAATCAGTAGGATATTAA

    GTAAAGATCATGTTCATGTTCATCAAGATATTTTGTGAATCTCCTACCGTAAATATATCAAAACATAATTTTTGATCACT
    GTAAAGATCATGTTCATGTTCATCAAGATATTTTGTGAATCTCCTACCGTAAATATATCAAAACATAATTTTTGATCACT

    AACATGCATTGCTAAGCATTCATTTGGAAAACTTCAAAGGTGATTTTCTCAGTATTTCGATTTTTTTTGCACCCTCAGAT
    AATATGCATTGCTAAGCATTCATTTGGAAAACTTCAAAGGTGATTTTCTCAGTATTTCGATTTTTTTGGCACCCTCAGAT

    TCCAGAGTTTCATTTATGAATGAGTTTAATAAATGAAGTTGGACAGAAAAAGTAAGGGAGTGTCGAGAGG
    TCCAGAGTTTCATTTATGAATGAGTTTAATAAATGAAGTTGGACAGAAAAAGTAAGGGAGTGTCGAGAGG
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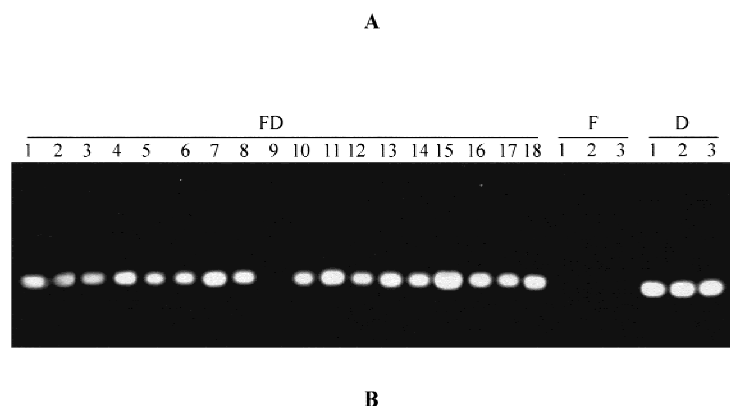
A



B

Fig. 7. Comparison of nucleotide acid sequences and PCR amplification of a shared fragment between the FD offspring and clone D. **A** The nucleotide acid sequences of the shared DNA fragment revealed by primer Opj-7 in the mated FD offspring (b) and in clone D (a). **B** Electrophoretogram of PCR amplified products in the mated FD offspring and clone D. A specific DNA fragment (indicated by the *arrow*) was amplified in FD offspring and in gynogenetic clone D by a pair of SCAR PCR primers that was designed according to the above nucleotide sequences, while no products were detected in gynogenetic clone F.

reproduction. In salmonid hybrids, uniparental chromosome elimination has been observed in early embryogenesis and is most likely involved in such hybrid inviability (Fujiwara et al. 1997). The present hypothesis suggests that the chromosome elimination occurring in the silver crucian carp should be different from that in inviable salmonid hybrids, because there is embryonic viability in the former. Therefore, the underlying mechanism of chromosome elimination remains to be determined.

In asexual species, the neutral mutations will pile up independently in each member of a chromosome pair, while in sexual species, over time those mutations will be lost by genetic shift, keeping chromosomes more similar. So asexual species should be highly divergent (Wuethrich 1998). Consistent with this view, a significant difference in nucleotide acid sequences was revealed in a shared DNA fragment between clone D and the mated FD offspring (Fig. 7A). It is known that polyploidization events have occurred in common carp and crucian carp, including the silver crucian carp, and generated duplicate copies of genes because of genome doubling. Rinsinger and Larhammar (1993) and Larhammar and Rinsinger (1994) have observed that in carp and crucian carp, duplicate genes can have unusual base replacements as well as inactivating mutations. From the viewpoint of sex evolution, the higher the mutation rate, the greater the advantage of sexual reproduction (Wuethrich 1998). With high divergence, once it has a chance to reproduce by gonochoristic reproduction, the silver crucian carp can obtain much more advantage and its offspring differentiate more acutely. Furthermore, silver crucian carp can remove harmful mutations and allow new combinations of favorable genes to come together by gonochoristic reproduction. On the other hand, it can break down the sterile obstacle of bisexual hybrids and overcome genetic separation in bisexual species by gynogenesis. In practice, the revelation of two reproduction modes has given us more suitable ways for selective breeding in the cultured silver crucian carp. We are able to obtain lots of genetic recombination offspring by gonochoristic reproduction, and we can select valuable clones with genetic stability by gynogenetic reproduction.

The response mechanism of two reproductive development modes might be the first discovery in vertebrates. Silver crucian carp, an excellent system with two reproductive development modes, can help to elucidate many questions in evolutionary biology, such as dispensable genes, sex origin and evolution, and genetic recombination. In other words, the evolutionary mechanism of gynogenetic vertebrates is far from being understood.

Acknowledgments. These studies were supported by the National Natural Science Foundation of China (Grants 39870430, 39730290, and 39823003), the Chinese Academy of Sciences (Grant KJ951-B1-607-03), and the Major State Basic Research Program (G1999053908) of the People's Republic of China.

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