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Genomic variation in the hybrids of white crucian carp and red crucian carp: evidence from ribosomal DNA

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In this study, we conducted a cross of white crucian carp (♀)×red crucian carp (♂) (WR), and characterized the morphology, reproduction and genetics of the progeny. Different from parents, WR with the gray color showed the hybrid morphological traits of both parents. WR possessed normal gonads producing mature eggs or sperm, and exhibited high fertilization rate (90.2%) and high hatchery rate (80.5%), which contributed to produce and enlarge the population. WR with the same DNA content as parents was a diploid fish with 100 chromosomes ($2n$=100). Amplified ITS of 45S rDNA, in WR the sequences consisting of 884 bp bases of the entire ITS-1 region, 5.8 S region, and entire ITS-2 region. The sequences showed high similarity between WR and its parents and leaned towards male inheritance. In WR, NTS of 5S rDNA consisted of three length types with total 654 bp bases. From sequence analysis of NTS, WR shared 94.2% and 95.1% similarities with their female and male parent, respectively. Sequence analysis of ITS and NTS revealed that there existed recombination and variation in the hybrid progeny, which was the genetic base for adaptation and speciation. In conclusion, we obtained WR from hybridization and it exhibited hybrid traits in morphology and variation in genetic composition showing essential difference with its parents. The obtainment of WR has important significance in fish genetic breeding.

white crucian carp, red crucian carp, hybridization, genomic variation

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Hybridization including distant crossing (interspecific or higher-ranking taxa hybridization) and intraspecific crossing involves the mating of genetically differentiated individuals or groups to generate new population with heterosis [1−3]. If the progeny derived from hybridization are bisexual and fertile, they can form new species or lineages with genetic variation through self-mating, with major implications for evolutionary biology and breeding. The application of hybrids in aquaculture is an important method and research content in fish genetic breeding. In our previous studies, many kinds of fishes were obtained from distant crossing, such as allotetraploid fish lineages derived from *Carassius auratus* red var. (♀)×*Cyprinus carpio* (♂) (F_3–F_{24}) [4], autotetraploid fish lineages derived from *Carassius auratus* red var. (♀)×*Megalobrama amblycephala* (♂) [5], diploid fish lineages derived from *Megalobrama amblycephala* (♀)×*Culter alburnus* (♂) (F_1–F_3) [6], different ploidy-level hybrids of *Megalobrama amblycephala* Yih×male *Xenocypris davidi* Bleeke [7] and different ploidy-level hybrids of red crucian carp×copmouth culter [8]. These hybrid progenies can be used to produce improved triploids or diploids [9,10]. In the theory, intraspecific crossing can also produce hybrid lineages with hetero-

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sis, which can be used in production after selection breeding. In aquaculture, there are few examples of establishment and implication of variation lineage by intraspecific crossing. In this study, we conducted a hybridization of white crucian carp (*Carassius auratus cuvieri*, WCC) and red crucian carp (*Carassius auratus* red variety, RCC). These are diploid fishes both belonging to Cypriniformes, Cyprinidae (genus *Carassius*) and with the same chromosome number (2*n*=100). WCC is characterized by white body color, high body, small head, short tail, strong reproductive ability, and quick growth. RCC is characterized by red body color, strong stress resistance, and perfect meat quality. However, WCC has the disadvantage of poor meat quality and RCC shows the disadvantage of slow growth. Besides, the red body color of RCC is restricted in terms of consumption. Thus from this crossing, we expect to obtain a new variety that has the combined advantage traits of WCC and RCC. In this study we obtained the hybrids of WR (F_1) from white crucian carp by red crucian carp and F_2 from F_1 inbred.

In this work, we characterized the morphological characteristics (countable and measurable traits) and gonad development of the hybrids. In addition, we investigated the cytogenetics of DNA content and chromosome number, and the molecular genetics of two multigene families 5S rDNA and ITS (from 45S rDNA) of WR. The ribosomal genes are divided into two clusters: the major (45S rDNA) and the minor (5S rDNA) clusters. The 45S rDNA has two internal transcribed spacers (ITS1 and ITS2), which are rapidly evolving DNA regions with high frequency of nucleotide mutations which make them useful as species-specific molecular markers. Meanwhile, the 5S rDNA comprises a conserved coding region of 120 bp and a non-transcribed spacer (NTS) which is variable in length and sequence among species. The two ribosomal clusters represent well-known examples of concerted evolution.

The results showed that the morphological traits of WR were intermediate between the paternal and maternal traits. The body color of WR was gray without red or white. WR possessed normal gonadal development. In cytogenetics level, the results showed 2*n* WR with 100 chromosomes. Sequence analysis of NTS and ITS revealed that gene recombination and base mutation happened in the hybrid progeny compared to its parents, which was the base for establishment of variation lineage. The heterosis of WR was just the needs of fish breeding. The formation and characterization of WR is important in biological evolution and fish genetic breeding.

1 Materials and methods

The samples including WCC and RCC were obtained from the Engineering Research Center of Polyploid Fish Breeding and Reproduction of Ministry of Education at Hunan Normal University.

1.1 Animals and crosses

During the reproductive seasons (from April to June), mature parental fishes were selected. The WR was formed by crossing haploid egg of WCC with haploid sperm of RCC. The progeny of reciprocal cross (RCC (♀)×WCC (♂), RW) was formed by crossing haploid egg of RCC with haploid sperm of WCC. Mature eggs were fertilized and the embryos developed in the culture dishes at the water temperature of 20–22°C. About 4,000 embryos were taken at random for the examination of the fertilization rate (number of embryos at the stage of gastrula/number of fertilized eggs×100%) and hatching rate (number of hatched fry/number of fertilized eggs×100%). The hatched fry were transferred to the pond for further culture.

1.2 Measurement of morphological traits

We randomly selected 20 one-year-old fishes from each group (WCC, RCC, WR and RW) for morphological examination. We measured total length, body length and height, head length and height, and caudal peduncle length and height (accurate to 0.1 cm) in each fish. These values were converted to ratios. In addition, we recorded counts of the following variables: number of lateral scales, number of upper and lower lateral scales, number of dorsal fins, number of abdominal fins, and number of anal fins. These data were analyzed by SPSS.

1.3 Examination of the ploidy level

We measured the DNA content of 20 WR and 20 RW individuals. The diploid WCC and RCC were used as control groups. We collected 0.5–1 mL blood from the caudal vein of each individual using a syringe containing ~200–400 U sodium heparin. The blood samples were then filtered, and stained with Cystain DNA 1 Step Staining Solution (Partec, Görlitz, Germany). The DNA content of each sample was measured by flow cytometry (Partec). The method of chromosome preparations referred to Luo [11].

1.4 Observation of the gonadal structure

To observe the gonadal structure, we selected eight 10-month-old WR and RW. The gonads were fixed in Bouin's solution for 24 h, then anhydrated using an ethanol gradient and transparentized in xylene. The sections were embedded in paraffin, cut at 6–8 μm, and stained with hematoxylin and eosin. The microstructure was observed and photographed using a Pixera Pro 600ES. We identified the gonad stages based on standards for cyprinid fish [12].

1.5 Genomic DNA extraction, PCR and sequencing

Total genomic DNA were extracted from the peripheral blood cells of RCC, WCC, RW and WR using a phenol/chloroform extraction method as described in Sambrook et al. [13]. Two sets of primers were used as described by Masaru Murakami [14]. 5S rDNA F: 5′-GCTATGCCCGATCTCGTCTGA-3′ and R: 5′-CAGGTTGGTATGGCCGTAAGC-3′; ITS F: 5′-AGTCGTAACAAGGTTTCCGTAG-3′ and R: 5′-GCTTA (G/C/A)TAATATGCTTAAATTC-3′) were designed and synthesized to amplify the 5S rDNA and ITS from genomic DNA. The amplification reaction mixture (25 μL) consisted of 20 ng genomic DNA, 1.5 mmol L^{-1} $MgCl_2$, 0.2 mmol L^{-1} of each dNTP, 0.4 μmol L^{-1} of each primer, 1× PCR buffer, and 1.25 U Taq polymerase (TaKaRa, Dalian, China). The thermal program consisted of an initial denaturation step of 94°C for 5 min, followed by 30 cycles (94°C for 30 s, 59°C for 30 s, and 72°C for 5 min) and a final extension step of 72°C for 10 min. The PCR products were separated on a 1.2% agarose gel, purified using a Gel Extraction Kit ((Sangon Biotech Co., Ltd., Shanghai, China), ligated into a pMD18-T vector, and transferred into *E. coli* DH5α. The positive clones were then sequenced (10 clones for each PCR fragment). To determine sequence homology and variation, the sequences were aligned using BioEdit [15] and Clustal W [16].

2 Results

2.1 Fertilization rates and hatching rates

In the reciprocal crossing and self-crossing of RCC and WCC, we observed high fertilization rates (WCC (♀)×RCC (♂): 90.2%, RCC (♀)×WCC (♂): 90.0%, RCC (♀)×RCC (♂): 92.1%, WCC (♀)×WCC (♂): 91.4%) and high hatching rates (WCC (♀)×RCC (♂): 80.5%, RCC (♀)×WCC (♂): 82.3%, RCC (♀)×RCC (♂): 84.5%, WCC (♀)×WCC (♂): 82.5%). There was no significant difference in fertilization or hatching rates between the hybrid groups and the parental self-crossing groups (P>0.05).

2.2 Comparison of morphological traits among RCC, WCC, RW, and WR

The hybrid progenies were all gray in body color with no red color (Figure 1). We produced F_2 which were all gray in body color without color differentiation. In this study we charged measurable characteristics into countable characteristics, reducing the influence caused by different size of fish. Table 1 presents the countable traits observed in WR, RW, WCC and RCC. Table 2 presents the measurable characteristics of WR, RW, WCC and RCC. RW and WR exhibited traits that were intermediate between the paternal and maternal traits.

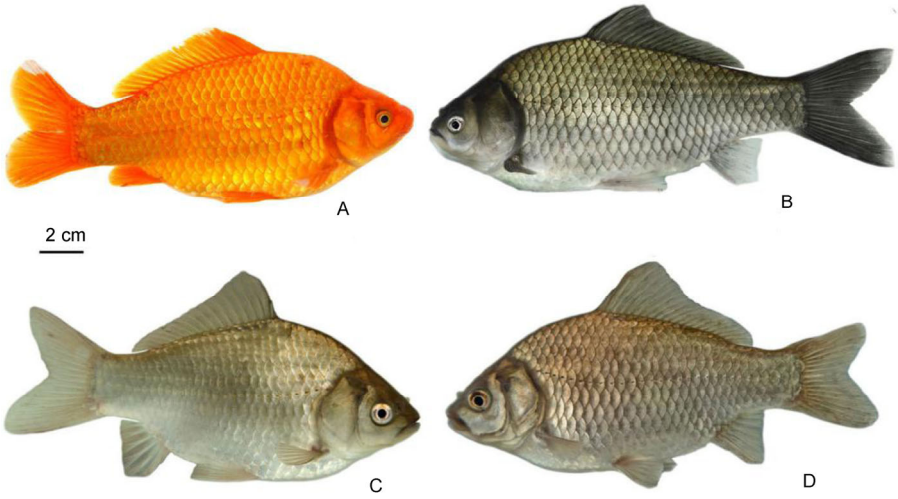

Figure 1 Images of RCC (A), WCC (B), RW (C), and WR (D).

Table 1 The ratios of measurable traits of RCC, WCC, WR and RW

Fish type	Total length/ body length	Body length/ body height	Body length/ head length	Head length/ head height	Caudal peduncle length/ caudal peduncle height	Head height/ caudal peduncle height
WCC	1.24±0.02	2.22±0.15	3.70±0.21	1.17±0.06	0.81±0.01	1.78±0.09
RCC	1.23±0.02	2.20±0.16	3.72±0.27	1.18±0.04	0.79±0.02	1.80±0.07
WR	1.25±0.03	2.20±0.15	3.70±0.25	1.19±0.06	0.82±0.02	1.81±0.12
RW	1.24±0.03	2.23±0.17	3.69±0.31	1.19±0.07	0.82±0.02	1.82±0.10

2.3 Ploidy levels of the hybrids

DNA content of WCC and RCC were used as control. The flow cytometry results indicated that the DNA content of the hybrids was the same as that of parental (P>0.05) (Figure 2), implying that these hybrids were diploid fish. Figure 3 shows the chromosome metaphase spreads. RW and WR possessed 100 chromosomes, indicating that they were diploid ($2n$=100).

2.4 Analysis of gonadal development of the hybrids

Histological analysis revealed that gonadal development in the hybrid was synchronized. Figure 4 presents the gonadal microstructure of 10-month-old WR and RW. The ovaries developed normally and were mainly composed of oocytes at phase IV-stage (Figure 4A and B). The testes of the hybrids were full of mature sperm (Figure 4C and D). Both RW and WR were sexually mature at one-year-old. White sperm or mature ova could be stripped out from 12-month-old hybrids.

2.5 Analysis of 5S rDNA and ITS

PCR amplification with 5S primers for RCC, WCC, WR and RW produced the same pattern with three bands (Figure 5). Sequencing revealed the DNA fragments to be 203, 340, and 471 bp in length. Using BLASTn, all fragments were confirmed to be 5S rDNA repeat units, each comprising a 3′ end of the coding region (positions 1–21), a whole NTS region, and a large 5′ coding region of the adjacent unit (positions 22–120). Three fragments of 5S rDNA (designated class I: 203 bp; class II: 340 bp; and class III: 471 bp) were categorized into different NTS types (designated NTS–I, II, and III for the 83, 220, and 351 bp monomers, respectively). The sequence data revealed that in the 5S coding regions, the parental samples RCC and WCC show species-specific nucleotide sites (position-22: RCC-G, WCC-T and position-

Table 2 Morphological assessment of RCC, WCC, WR and RW

Fish type	Lateral scale	Upper lateral scale	Lower lateral scale	Back fin	Abdominal fin	Anal fin
WCC	32–34	6–8	5–7	III+18–20	8–10	III+6–7
RCC	28–30	5–6	6–7	III+18–20	8–9	III+6–7
WR	30–32	5–7	6–7	III+18–20	8–10	III+6–7
RW	30–32	6–8	5–7	III+18–20	8–10	III+6–7

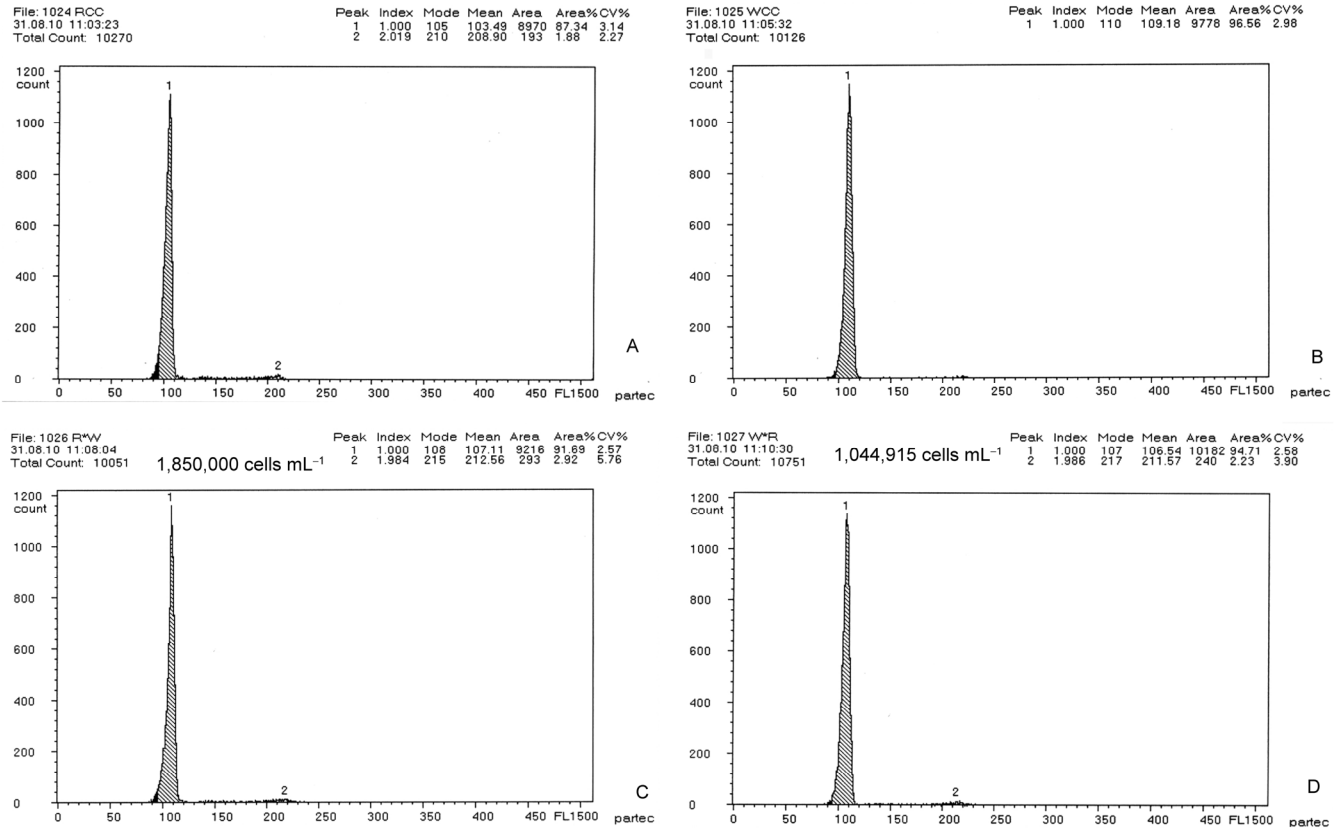

Figure 2 The DNA content in RCC (A), WCC (B), RW (C), and WR (D).

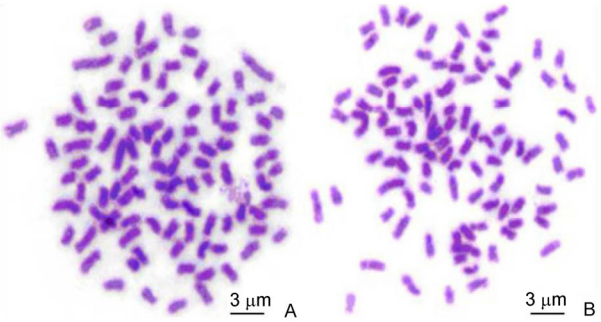

Figure 3 The metaphase chromosome spreads of RW and WR. A, The metaphase chromosome spread of RW ($2n$=100). B, The metaphase chromosome spread of WR ($2n$=100).

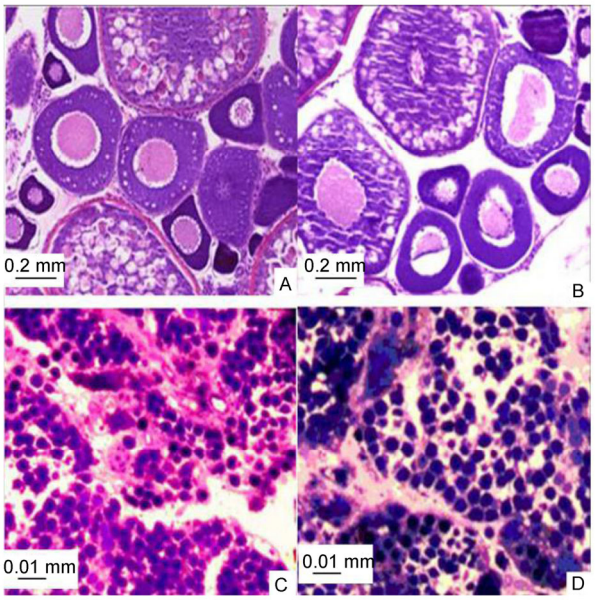

Figure 4 Gonad microstructure of RW and WR. The mature ovary of RW (A) and WR (B). The mature testis of RW (C) and WR (D).

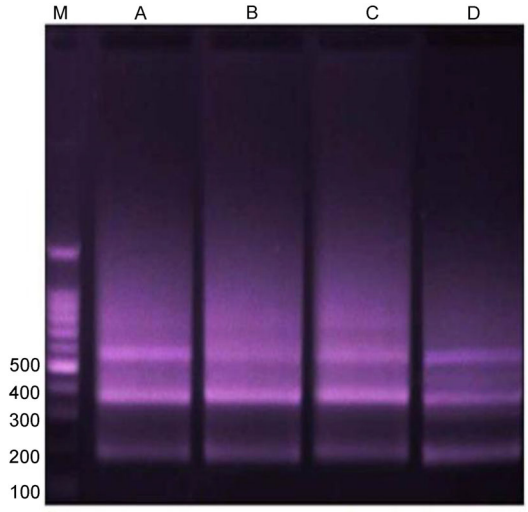

Figure 5 DNA bands amplified from RCC, WCC, RW, and WR. M: DNA 100 bp ladder; 5S amplification resulted in three DNA bands (~200, 350 and 500 bp) from RCC (A), WCC (B), RW (C), and WR (D).

66: RCC-G, WCC-A). The progenies RW and WR inherited at position-22 the WCC nucleotide with T and at position-66 their respective male-parent nucleotide (RCC: G, WCC: A, RW: A, WR: G) (Figure 6). In the full length of NTS, the sequence similarities of WR with WCC and RCC were 94.2% and 95.1%, respectively. Comparative analysis of the NTS between the hybrid offspring and their parents showed obvious recombination and base mutation. In NTS-83, the nucleotide of WR was different from its parents at seven sites (position-5, -9, -12, -17, -24, -25 and -33) (Figure 7), revealing large variations. Table 3 shows the bases in the hybrid offspring that were different from the parents. In the NTS-220, sequence from the hybrid progenies exhibited influence by RCC (Figure 8), whereas in the sequence NTS-351, sequence from the hybrid progenies exhibited influence by WCC (Figure 9) and WR displayed larger variation than RW (Table 3).

PCR amplification, sequencing and sequences analysis of the 45S rDNAs of WCC, RCC, WR and RW revealed that these were of the same length (884 bp) with ITS1 (341 bp)+5.8S (159 bp)+ITS2 (384 bp) showing above 99% sequence similarity (Table 4). In the 5.8S rDNA, we observed one nucleotide difference at position -158 (RCC: T, WCC: C, RW: C, WR: T) (Figure 10). In ITS1 region, we observed two nucleotide differences at positions-90 (RCC: A, WCC: C, RW: C, WR: C) and -309 (RCC: A, WCC: G, RW: G, WR: A) (Figure 11). In ITS-2 region, we observed four nucleotide differences at positions-141 (RCC: T, WCC: –, RW: –, WR: –), -325 (RCC: –, WCC: –, RW: –, WR: T), -349 (RCC: –, WCC: C, RW: C, WR: –) and -351 (RCC: C, WCC: A, RW: A, WR: C) (Figure 12). In Table 4, the hereditary characteristics of ITS were inclined to paternal inheritance.

3 Discussion

Hybridization, as a means of species modification, plays a very important part in the speciation and improvement of animals [17–19]. It integrates the genetic material of both parent species into one single group, resulting in changes in the gene regulation and expression in the hybrids [18,20,21].

Table 3 Similarity of NTS sequences between hybrids and their parents

NTS type	WR/WCC	WR/RCC	RW/RCC	RW/WCC
NTS-83 bp	91.6%	89.2%	98.8%	98.8%
NTS-220 bp	94.5%	99.1%	99.1%	94.5%
NTS-351 bp	98.0%	96.9%	96.3%	99.1%

Table 4 Similarity of ITS sequences between hybrids and their parents

ITS type	WR/WCC	WR/RCC	RW/RCC	RW/WCC
ITS-1	99.7%	99.7%	99.4%	100%
ITS-2	99.2%	99.5%	99.2%	100%

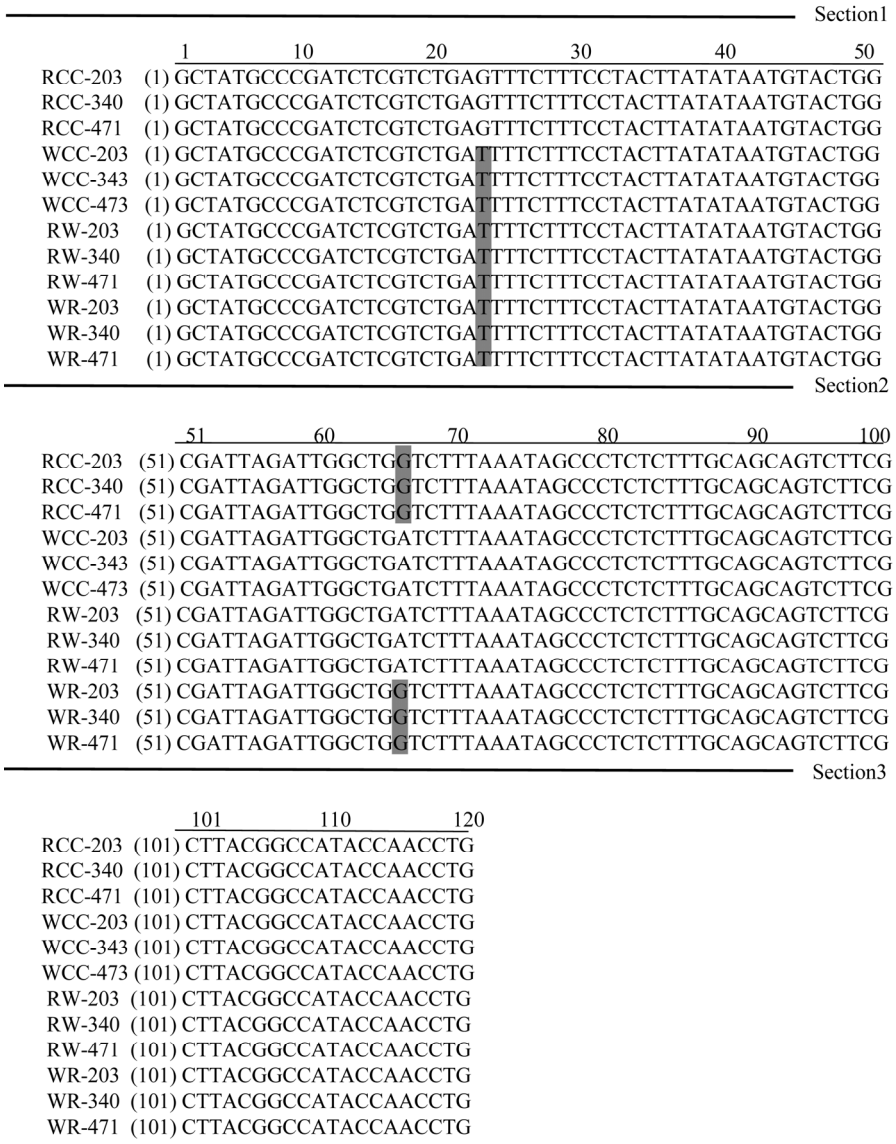

Figure 6 Comparison of the 5S coding regions from RCC, WCC, RW, and WR.

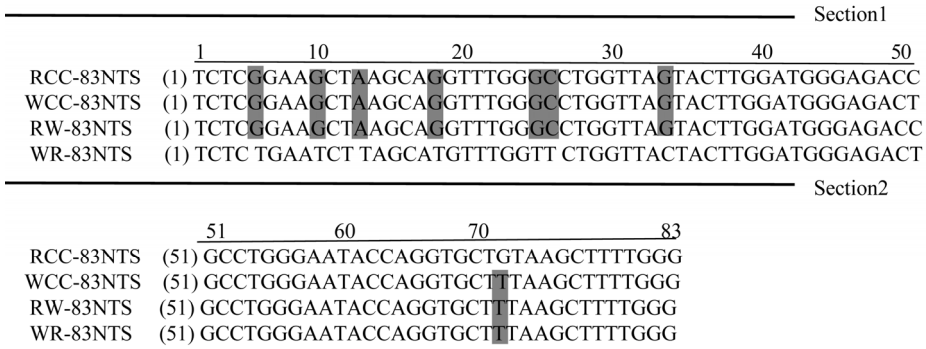

Figure 7 Comparison of the NTS-83 sequences from RCC, WCC, RW, and WR.

Thus, the hybrids gain heterosis in many aspects, such as growth rate, desirable traits, harvestability, and environmental tolerances [1,22]. The two kinds of hybridization are distant crossing (above-specific or interspecific crossing) and intraspecific crossing. Through long-term and systematic research in distant hybridization, our lab cultivated a lot

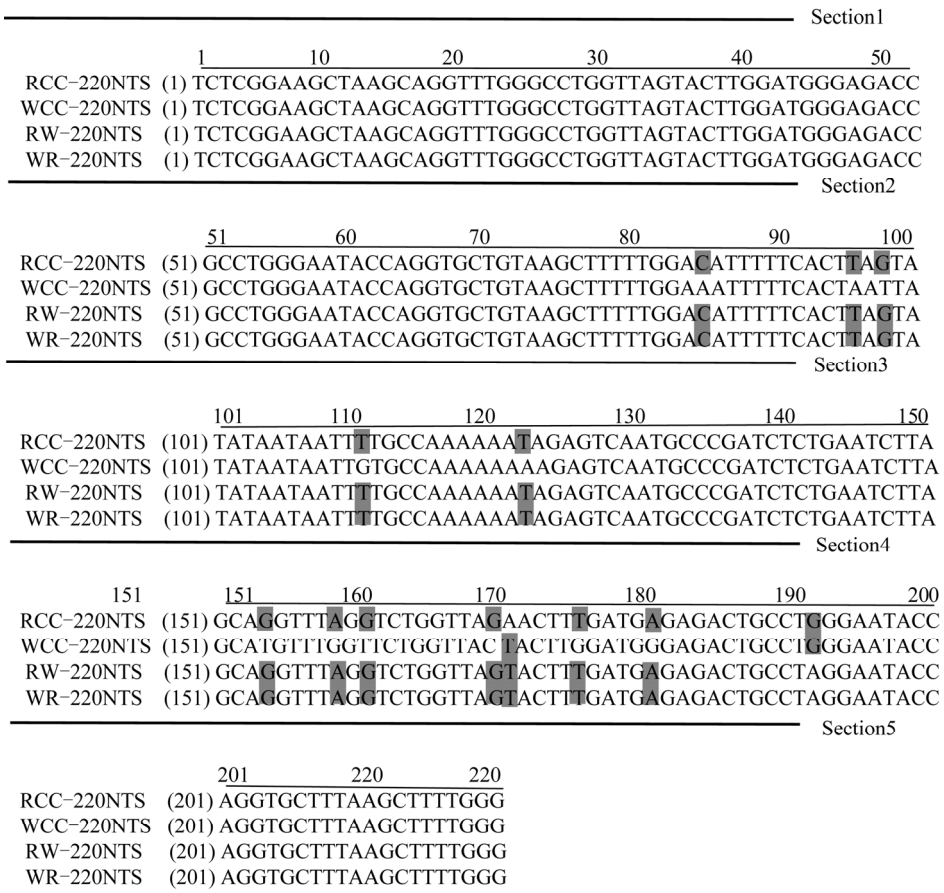

Figure 8 Comparison of the NTS-220 sequences from RCC, WCC, RW, and WR.

of excellent new strains successfully, such as allotetraploid fish lineages derived from *Carassius auratus* red var. (♀)×*Cyprinus carpio* (♂) (F_3–F_{24}), autotetraploid fish lineages derived from *Carassius auratus* red var. (♀)× *Megalobrama amblycephala* (♂), diploid fish lineages derived from *Megalobrama amblycephala* (♀)×*Culter alburnus* (♂) (F_1–F_3). At the DNA level, distant hybridization leads to the emergence of recombinant DNA variation in the offspring. With regard to phenotype, distant hybridization integrates the advantages of both parents, so that heterosis of shape, growth rate, survival, disease resistance, and other traits is exhibited by future generations [23]. An in-depth study of fish chromosome evolution, unreduced gamete formation mechanism, polyploidy, gene recombination and variation can be continued on the basis of the distant hybridization above. Distant hybridization offspring have broader applications in production, such as the production of new polyploid fish, sterile triploid fish and the new diploid fish. The formation of distant hybrid lineages is very useful for studies of biological evolution and genetic breeding.

Intraspecific hybridization defined as a cross between two subspecies or different strains in same species. Because of the close relationship, hybrid lethality and intersterility will not appear in intraspecific hybridization. Although intraspecific hybridization brings finite genetic variation than distant hybridization in hybrids, as long as matches appropriately, intraspecific hybridization can also produce the offspring possessing the excellent traits generated from parents. Furthermore, it is easy for intraspecific hybridization to build a steady lineage. The genetically stable hybrids can not only transmit the heterosis generation by generation, but also produce various new strains or improve fish by match with the same or different ploidy fish. Thus, intraspecific crossing is indispensable to fish genetic improvement.

The choice of parents for hybridization experiments requires consideration of chromosome number, phylogenetic relationships, reproductive characteristics, shape, diet, growth rate, disease resistance, body color and other factors. When designing hybrid crossing, parents possessing different desirable traits are chosen to produce hybrids with a combination of excellent hybrid characteristics. In this paper, we carried out the crossing of white crucian carp and red crucian carp which possess the same number of chromosomes, different color, and different excellent traits. Both of white crucian carp and red crucian carp belong to the order Cypriniformes, genus *Carassius.* Previously, white

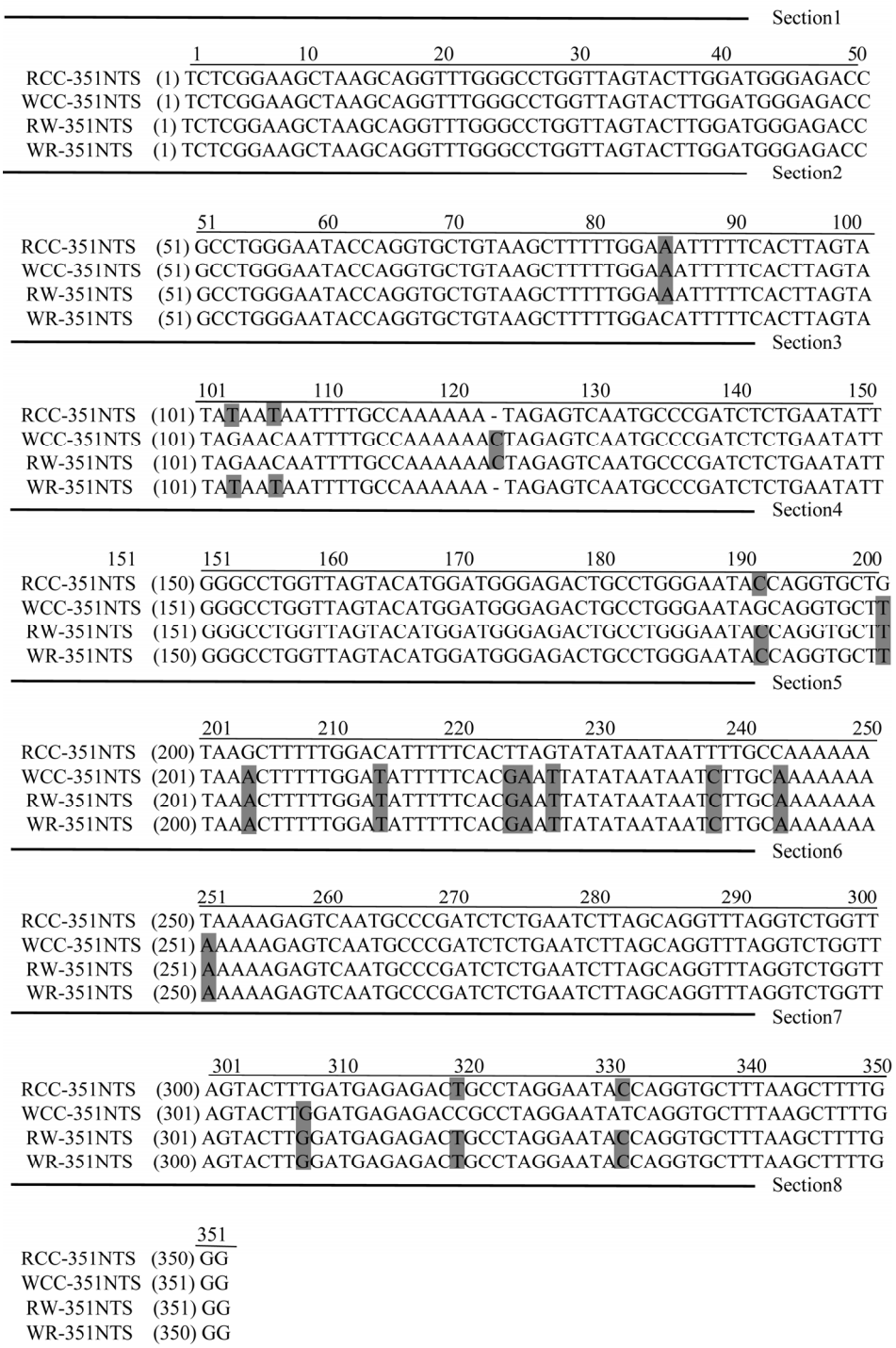

Figure 9 Comparison of the NTS-351 sequences from RCC, WCC, RW and WR.

crucian carp had been treated as a species, *C. Carassius*, the same as red crucian carp. However, based on ample molecular evidence recently, white crucian carp is considered to be an independent species, *Carassius cuvieri* [24–26]. Therefore, the crossing of white crucian carp and red crucian carp can also be considered as a distant hybridization at the interspecific level. The degree of heredity and heterosis was assessed from shape, fertilization rate, hatching rate, gonadal development, ploidy level, 5S rDNA and ITS of parental fishes and hybrids. In this hybridization, we observed phenotypic alteration in the hybrids. The hybrids' measurable characteristics and countable characteristics are between parents'. The hybrid progenies were all gray in body color without red color. The hybrids exhibited high fertilization and hatching rates, which were close to that of the parental self-mating. The hybrids all reach sexual ma-

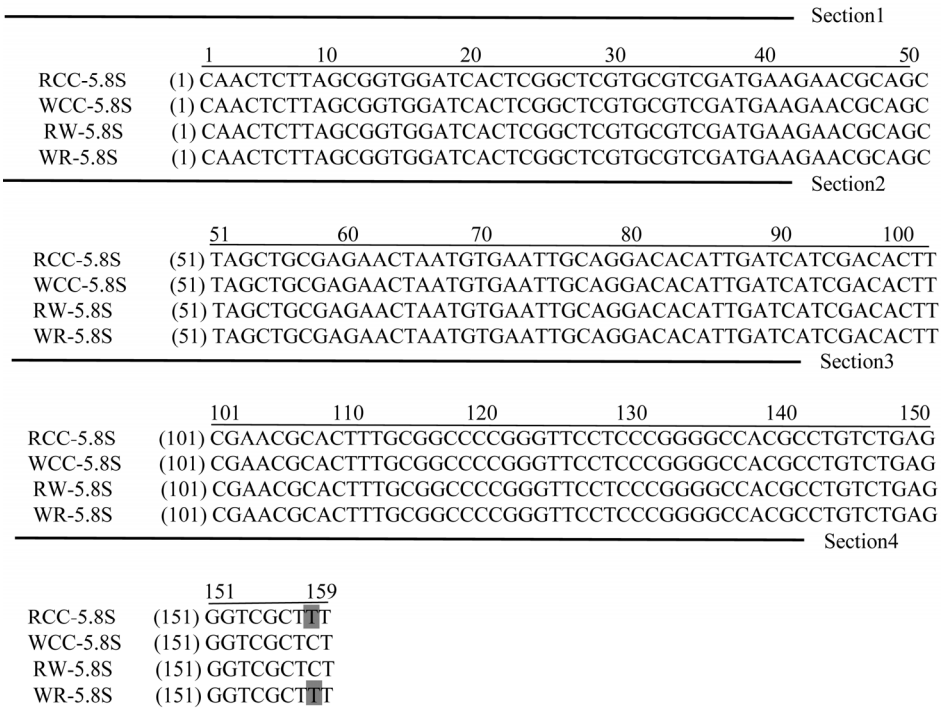

Figure 10 Comparison of the 5.8s rDNA sequences from RCC, WCC, RW, and WR.

Table 5 Nucleotide changes in NTS and ITS[a)]

Fish type	Sequence type	Sequence length (bp)	Change base#	Mutation base#	Female-specific base#	Male-specific base#
RW	NTS-83	83	2	0	1	1
	NTS-220	220	13	1	11	1
	NTS-351	351	16	0	3	13
	Total		31	1	15	15
WR	NTS-83	83	9	7	2	0
	NTS-220	220	13	1	1	11
	NTS-351	351	17	1	10	6
	Total		39	9	13	17
RW	ITS-1	341	2	0	0	2
	ITS-2	384	3	0	0	3
	Total		5	0	0	5
WR	ITS-1	341	2	0	1	1
	ITS-2	384	4	1	1	2
	Total		6	1	2	3

a) # represents number.

turity in one year, and WR had been successfully developed F_2 generation, which charged the traditional view of distant hybrids' infertility or sterility. This was an advantage impossible in other distant crossing. Flow cytometry and chromosome preparation confirmed that the hybrids are diploid fish without polyploid progeny. This is different from some interspecific crossing which results in polyploidization in the hybrid progeny [3,4,27].

In molecule genetics, ribosomal DNA was studied. The moderate conserved regions of NTS and ITS show quick evolution speed, and these are used to analyze evolution and genetic relationship. In this study, the sequence of NTS in the hybrids of WR and RW exhibited obvious changes that can be categorized into three: (i) hybrids heredity female parent (15 sites in RW and 13 sites in WR), (ii) heredity male parent (15 sites in RW and 17 sites in WR), and (iii) base mutated (seven sites in WR-NTS-83, one site in WR/RW-NTS-220, one site in WR-NTS-351) (Table 5). This indicated that the hybridization resulted in gene recombination and base mutation in the hybrid progeny. ITS

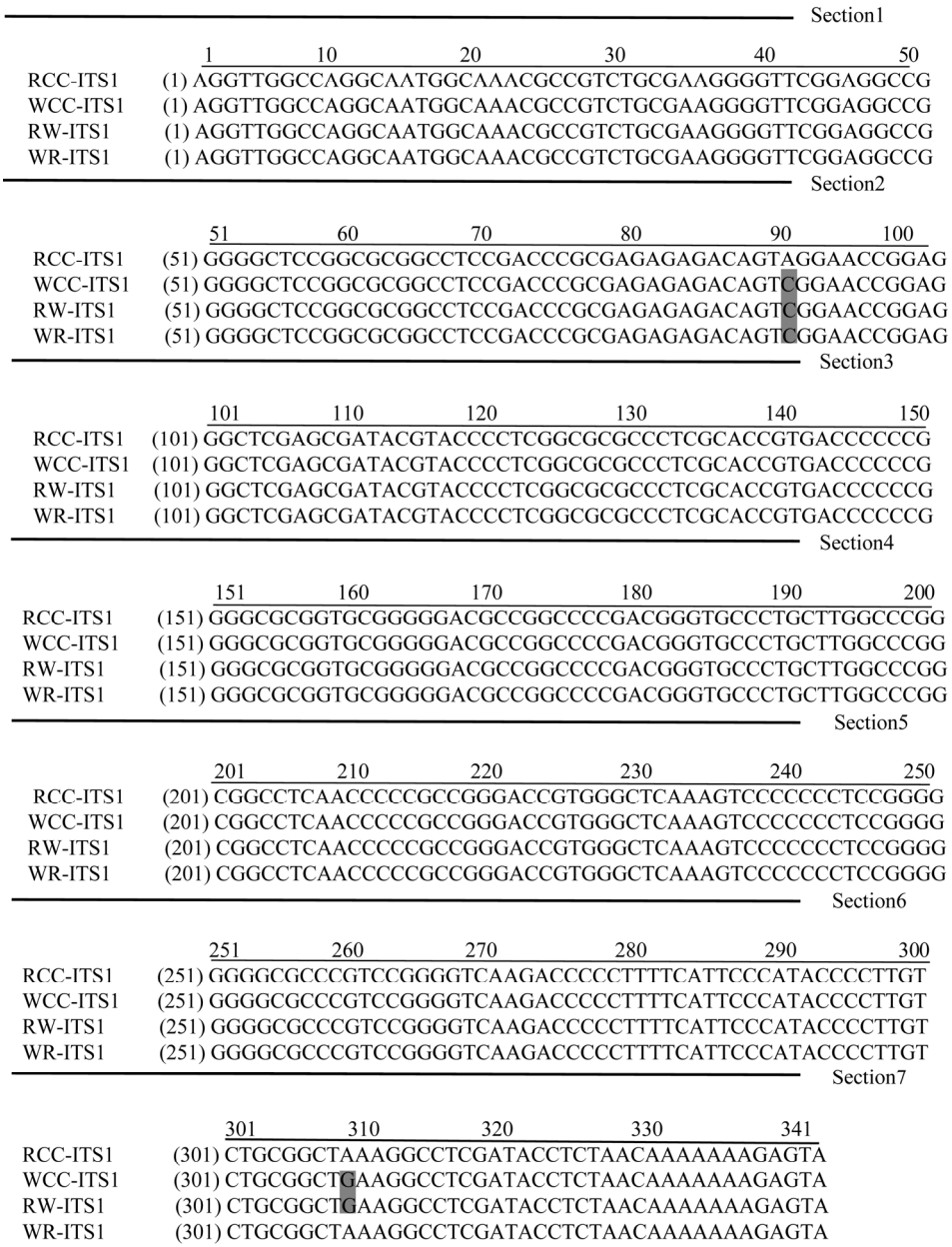

Figure 11 Comparison of the ITS1 sequences from RCC, WCC, RW, and WR.

sequence analysis also showed that genetic recombination and base mutation happened in WR. Table 5 lists the base variation in NTS and ITS of WR and RW. Recombination of nuclear DNA in hybrids plays an important role in hybrid fish lineages formation and molecular evolution, which is one of the important genetic variation characteristics. It makes the hybrids adapt to the environment, survive and multiply sustainably in the hybrid genetic background. In both NTS and ITS, the base variation in WR showed more frequently than that in RW (Table 5). RW exhibited 100% similarity in ITS sequence with the male parent of WCC. In WR, there were three types of specific base observed (female parent specific base, male parent specific base and mutation base), leaning towards paternal inheritance.

In morphology, there was no significant difference between WR and RW. At the genomic level, however, significant differences became apparent in WR showing larger variation than RW. The reason and more various genetic traits of WR are being researched. Nuclear DNA mutation and recombination were detected in WR, which was the basis for phenotypic changes. WR inherited both female advantages like rapid growth rate and favorable shape and male advantages like perfect meat quality and strong disease resistance. We obtained F_2 population without characteristic segregation from F_1 self-crossing, which indicated the heterosis in WR could be inherited steadily. Therefore, WR possessed the necessary conditions to form a new genetic variation lineage: (i) nuclear DNA recombination and varia-

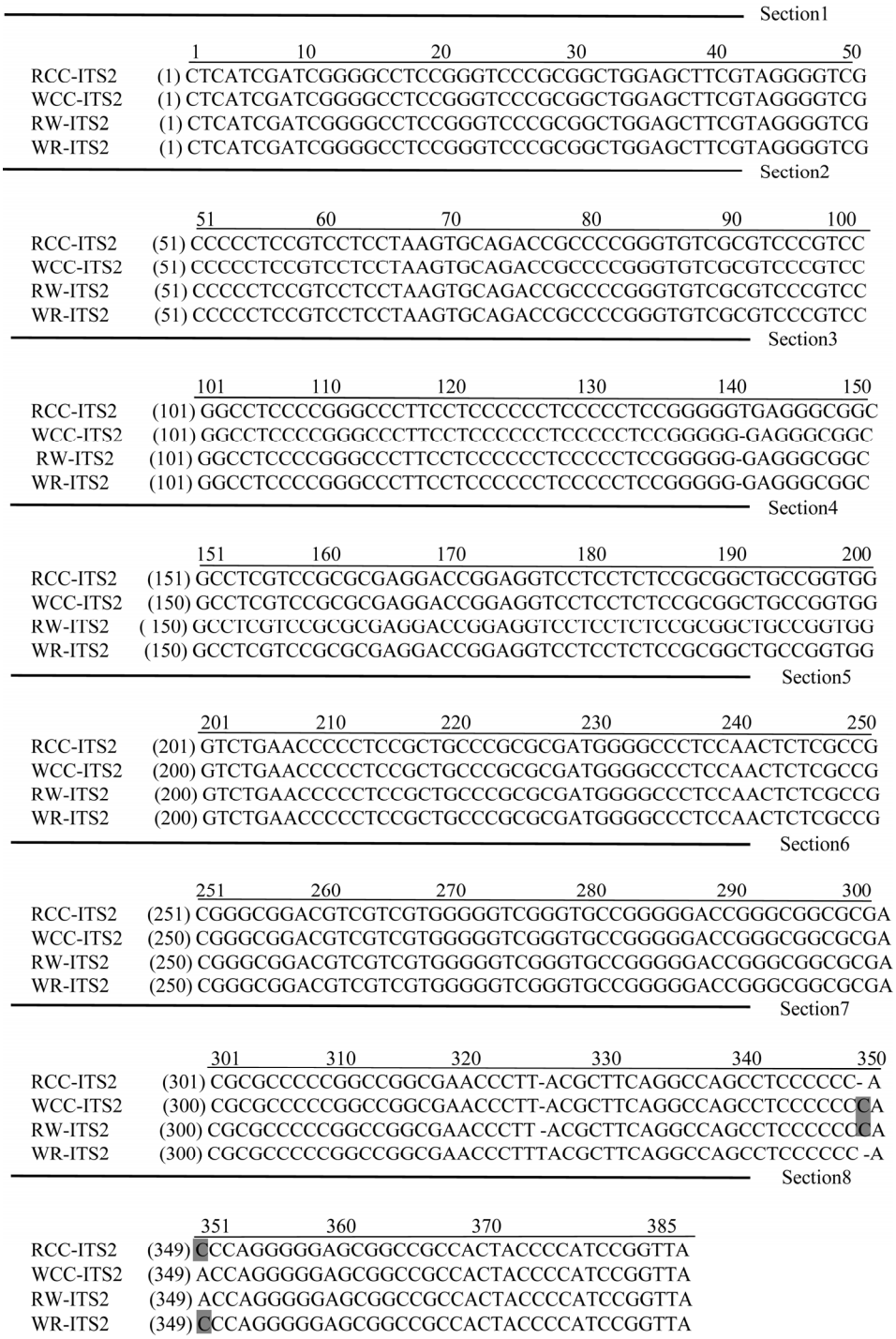

Figure 12 Comparison of the ITS2 sequences from RCC, WCC, RW, and WR.

tion; (ii) effective integration of parent advantages; (iii) bisexual fertile and the heterosis inherited stability. In conclusion, characterization of WR provided new insights into the genotypic variation and phenotypic diversity of fish hybrids obtained from hybridization. In our previous study, both RCC and WCC were commonly used as the female parent to mate with tetraploids in order to produce triploids in aquaculture. The WR gained good characteristics from both RCC and WCC, bringing a new germplasm to replace WCC and RCC to produce triploid, which will benefit to aquaculture. It is of important significance not only to work on fish breeding, but also to research the genomic variation in hybridization.

The authors declare that they have no conflict of interest. All applicable institutional and national guidelines for the care and use of animals were followed.

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