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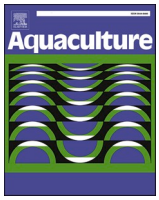

An improved autotetraploid fish derived from the hybridization of gynogenetic homologous diploid crucian carp (♀) × autotetraploid *carassius auratus* (♂)

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ABSTRACT

The autotetraploid *Carassius auratus* (4nRR, 4n = 200) produces stable diploid gametes and has been maintained as a genetically stable lineage (F_2–F_{20}). This process further facilitates the generation of all-female autodiploid gynogenetic offspring (G_1) (2n = 100, RR), which produce stable diploid gametes. In this study, a diploid gynogenetic clonal hybrid line (G_1–G_6, 2n = 100) was established via gynogenesis, and an improved autotetraploid *Carassius auratus* (4nRG, RRRR, 4n = 200) was subsequently produced by hybridizing 4nRR with the G line. The morphological traits, ploidy level, and reproductive biology traits of 4nRG were subsequently investigated. No significant differences ($p > 0.05$) were observed between 4nRG and 4nRR in morphological traits. Moreover, DNA content and chromosome number analyses confirmed 4nRG as a tetraploid. Histological sections revealed normal gonadal development in both female and male 4nRG individuals. Microstructural and ultrastructural analysis revealed that the 4nRG sperm developed normally with intact head-tail structures, and the oocytes exhibited diameters comparable to those of 4nRR. Importantly, under controlled conditions, assessments of sperm motility, gonadosomatic index (GSI), relative fecundity, relative milt volume, fertilization rate, hatching rate, and survival rate collectively demonstrated that 4nRG exhibited significantly superior reproductive performance compared to the 4nRR. The newly developed 4nRG exhibited robust reproductive capabilities, offering a novel gamete resource for polyploid breeding.

1. Introduction

Polyploidization, or whole-genome duplication, is a widespread natural phenomenon that results in the duplication of genes within an organism's genome. It is widely recognized as a key driver of evolution and biodiversity in many plant and animal species (De Wet, 1979; Otto and Whitton, 2000; Wolfe, 2001). Polyploids are classified based on the origin of chromosome doubling, with two main categories: autopolyploids, derived from a single taxon, and allopolyploids, formed through the merging of chromosome sets from different species (Chen, 2007; Song et al., 2012). In aquaculture, polyploid breeding has long been a topic of considerable interest. Extensive research has focused on the induction of tetraploid fish lines using various techniques, such as cytochalasin B, hydrostatic pressure, and heat/cold shocks. Despite these efforts, the establishment of stable tetraploid lines remains challenging, with only a few successful cases reported (Chourrout et al., 1986; Hershberger and Hostuttler, 2007; Refstie, 1981; Zou et al., 2004). However, distant hybridization has proven to be an effective strategy for generating genetically stable tetraploid lineages. Furthermore, this approach facilitates the large-scale production of enhanced triploid fish through interploid hybridization between tetraploids and diploids (Liu, 2010; Xu et al., 2015).

Previous studies have successfully generated allotetraploid hybrids (F_1, 4n = 148, RRBB) through distant hybridization between *Carassius*

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auratus red var. (RCC, 2n = 100, RR, ♀) × *Megalobrama amblycephala* (BSB, 2n = 48, BB, ♂). It is now understood that chromosomal abnormalities during meiosis in these allotetraploid hybrids yield diploid gametes (sperm and eggs). Upon fertilization, these gametes result in the production of autotetraploid *Carassius auratus* (4n = 200, 4nRR) in the F_2 generation (Qin et al., 2014b). The absence of homologous chromosome pairing in allopolyploids results in the formation of bivalents during gamete production, which ultimately yields diploid gametes (Liu et al., 2006; Ramsey and Schemske, 2002; Zhang et al., 2005). Bivalent pairing is regarded as the most effective mechanism for maintaining genetic stability in polyploids. In contrast, homopolyploids frequently encounter disruptions in homologous chromosome pairing during meiosis, which can impede the formation of typical diploid gametes and lead to reduced fertility or even sterility (Deniz, 2002). Consequently, the reproductive ability of homopolyploids is often questioned. Interestingly, both male and female autotetraploids, including those from the F_2 and subsequent generations, can produce unreduced diploid gametes (Qin et al., 2014a). Fertilization involving these diploid gametes gives rise to 4nRR individuals, with the autotetraploid line successfully propagated across successive generations from F_3 to F_{20}.

Gynogenesis is a unique reproductive approach analogous in principle to distant hybridization, which involves the inactivation of paternal genetic material through specific treatments to induce egg development, resulting in an offspring that carries genetic information almost exclusively from the mother (Eeckhaut et al., 2001; Manan et al., 2022; Sun et al., 2006; Xu et al., 2015). Traditionally, gynogenetic offspring have been considered predominantly or entirely female, with genotypes highly similar to those of the maternal parent. However, increasing evidence suggests that some gynogenetic progeny exhibit male phenotypes or paternal traits, which may result from the partial integration of paternal DNA during the gynogenetic process, an effect referred to as the "allo-sperm effect" (Liu et al., 2025). In this study, a diploid gynogenetic clonal hybrid crucian carp line (G_1–G_6, 2n = 100, RR) was established through successive generations of artificial gynogenesis. The first generation (G1) was produced from the eggs of 4nRR females activated by UV-irradiated BSB sperm. This G line is capable of producing unreduced diploid gametes, which allows its propagation through consecutive gynogenetic generations. Subsequently, each successive generation (G_2–G_6) was obtained by inducing gynogenesis using eggs from the previous generation activated by UV-irradiated BSB sperm, without the need for chromosome-doubling treatment (Qin et al., 2018).

Following the successful development of an autotetraploid fish line (4nRR, 4n = 200, F_2–F_{20}) and the G line (G_1–G_6, 2n = 100), an improved autotetraploid fish (4nRG, 4n = 200) was artificially generated via hybridization between G (♀, 2n = 100) and 4nRR (♂, 4n = 200). In this study, the morphological traits, chromosome number, DNA content, gonadal development, gamete morphology, and reproductive performance of the 4nRG individuals were systematically examined. This research is significant for the advancement of fish genetic breeding and our understanding of species evolution.

2. Materials and methods

2.1. Ethics statement

In accordance with Chinese animal research guidelines, formal approval from regulatory agencies was not required as the experimental fish species are not on any national protected species lists. All specimens were reared in natural pond environments and sacrificed humanely with 2-phenoxyethanol (Sigma) before subsequent analyses.

2.2. Animals and crosses

The fish utilized in this research were provided by Hunan Normal University's State Key Laboratory of Developmental Biology of Freshwater Fish in China. In our previous study, we obtained 4nRR (4n = 200) via RCC (♀, 2n = 100) × BSB (♂, 2n = 48). Subsequently, the gynogenetic offspring G_1 was generated from the eggs of 4nRR activated by UV-irradiated BSB sperm. The G line was then established by a second round of gynogenesis from G_1 eggs using UV-irradiated BSB sperm.

During the 2024 reproductive season (April–June), eggs from G_5 crucian carp were activated using UV-irradiated BSB sperm to induce artificial gynogenesis, following the protocol described by Qin et al. (2018), resulting in the G_6 generation. Subsequently, 10 G line females were crossed with 10 4nRR males to produce 4nRG offspring, while 10 4nRR self-crosses served as the control. To induce ovulation, fish were injected intraperitoneally with a hormone mixture consisting of 200 IU/kg human chorionic gonadotropin (HCG), 10 μg/kg luteinizing hormone-releasing hormone A2 (LRH-A2), and 1 mg/kg domperidone. Once spawning behavior was observed, the females were stripped for artificial insemination. Approximately 200,000 eggs from G line females were fertilized with sperm from 4nRR males to produce 4nRG offspring, while a separate batch of 200,000 eggs from 4nRR females was fertilized with 4nRR sperm as the control. Mature eggs were fertilized, and the embryos hatched in the culture dishes at a water temperature of 24–26 °C. Since the eggs of 4nRR and G fish were demersal without stickiness, desticking was omitted. The fertilization rate (no. embryos at the gastrula stage/no. eggs) and hatching rate (no. hatched fry/no. eggs) were calculated for 6000 embryos. Subsequently, all fry were transferred to an aerated earthen pond (approximately 200 m^2 in area and 1.5 m in depth) supplied with continuous flowing freshwater. The pond was maintained under natural photoperiod conditions, and water temperature was kept between 24 °C and 30 °C during the rearing period. Fry were fed commercial feed three times daily until they reached the juvenile stage.

2.3. Measurement of morphological traits

1-year-old 4nRG (n = 10) and 4nRR individuals (n = 10) were randomly selected for morphological analysis. The following measurable traits were recorded: whole length (WL), body length (BL), head length (HL), caudal peduncle length (CPL), body height (BH), head height (HH), and caudal peduncle height (CPH). These measurements were converted into proportional values to normalize for body size differences. Countable traits included the number of lateral line scales, upper and lower lateral scales, and the number of fin rays in the dorsal, pelvic, and anal fins.

2.4. Analysis of ploidy level

The ploidy level of 4nRG individuals was assessed by quantifying the mean DNA content of erythrocytes and analyzing metaphase chromosome numbers. DNA content in erythrocytes from 4nRG, 4nRR and G_6 was quantified using a flow cytometer (Cell Counter Analyzer, Partec). Approximately 0.5 mL of blood was collected from the caudal vein and immediately transferred into syringes containing 100–200 units of sodium heparin to prevent coagulation. Blood samples were processed according to previously described protocols (Liu et al., 2007a, 2007b). Chromosome spreads were prepared from the kidney tissues of 1-year-old 4nRG, 4nRR, and G fish. The preparations followed the procedure described by (Xiao et al., 2014), and chromosome morphology and numbers were analyzed under a light microscope.

2.5. Gonadal structure, fecundity, sperm volume, and gonadosomatic index (GSI)

At one year of age, 20 individuals from each of the 4nRG and 4nRR groups were randomly selected for histological examination of gonadal development. Gonadal tissues were fixed in Bouin's solution, dehydrated, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). The stained sections were observed and photographed

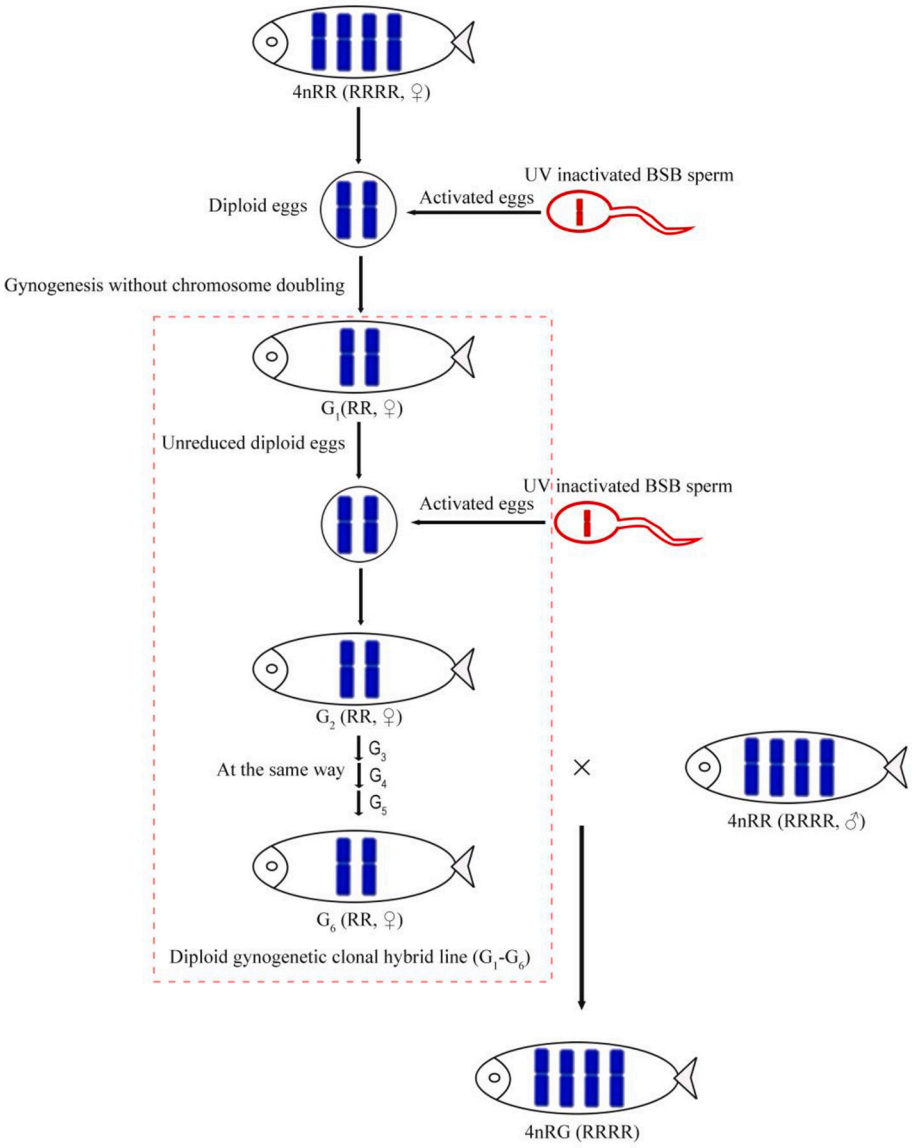

Fig. 1. Schematic diagram of the experimental fish generation.

under an Olympus CX41 light microscope. The stages of gonadal development were determined based on (Sun et al., 2003) cyprinid criteria.

Absolute fecundity was determined from dissected ovaries by counting hydrated oocytes in a weighed tissue sample of approximately 1 g taken from the central ovarian region and fixed in formalin, and then extrapolating the count to the total egg number based on the total ovarian weight. Relative fecundity (eggs·g^{-1} body weight) was calculated by dividing the estimated total egg number by the total body weight. For milt volume analysis, milt was collected from males via gentle abdominal stripping without anesthesia to avoid contamination from urine or water. Absolute milt volume was recorded immediately using a graduated micropipette with 0.01 mL precision. Relative milt volume (mL·g^{-1} body weight) was calculated accordingly. All samples were kept on ice until further processing. The GSI was evaluated for both 4nRR and 4nRG. Fish were deeply anesthetized with 100 mg/L MS-222, weighed, and dissected to remove the gonads and visceral organs. The GSI was calculated using the formula: GSI = 100 × gonad weight / somatic body weight, where somatic body weight refers to the total body weight minus the weight of visceral organs (Lee et al., 2025).

2.6. Electron microscopy of testicular and sperm ultrastructure

Oocytes from 1-year-old 4nRG and 4nRR females were observed under a stereomicroscope to examine their morphological characteristics. Mature eggs were placed in Petri dishes with clean water and imaged using a stereo zoom microscope (Olympus SZX16, Japan). Egg diameter was measured using ImageJ software (NIH, USA), and the average values were recorded for each group.

For male samples, testicular tissue from 4nRG and 4nRR individuals was collected and immediately fixed in 2.5 % glutaraldehyde prepared in 0.1 M phosphate buffer (pH 7.4) for 4 h at 4 °C. Following post-fixation in 1 % osmium tetroxide for 2 h at room temperature, the samples were rinsed with phosphate buffer, dehydrated through a graded acetone series, and embedded in Epon 812 resin. Ultrathin *sec*tions (70–90 nm) were prepared using a Leica ultramicrotome, stained with uranyl acetate and lead citrate, and examined using a Hitachi HT7800 transmission electron microscope (Hitachi High-Technologies, Japan) at 80 kV.

Semen was collected from 1-year-old 4nRG and 4nRR males and fixed in 2.5 % glutaraldehyde for over two hours. A 10 μL aliquot was air-dried on clean slides, stained with Giemsa solution (Solarbio, China)

Table 1
Fertilization, hatching, and survival rates of 4nRR and 4nRG fish[1].

Fish type	Egg source	Sperm source	Fertilization rate(%)	Hatching rate(%)	Survival rate(%)
4nRG	G	4nRR	80.23 ± 3.25[a]	65.34 ± 3.09[a]	61.68 ± 2.56[a]
4nRR	4nRR	4nRR	70.43 ± 2.13	56.12 ± 3.41	52.78 ± 2.67

[1] Values were reported as mean ± SEM ($n = 10$). [a] Values with different letters within the same column are significantly different ($p < 0.05$).

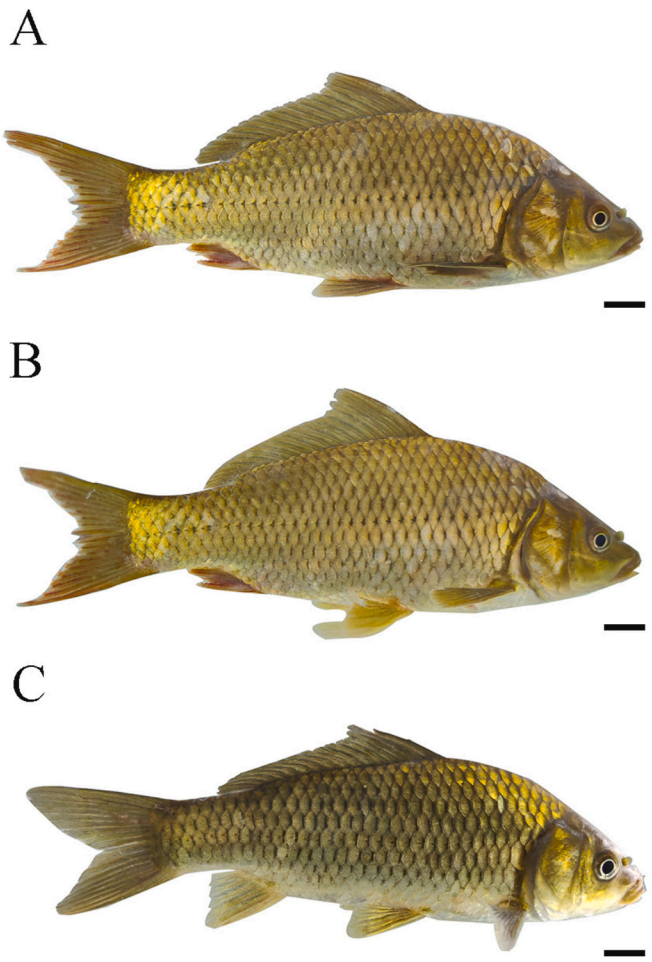

Fig. 2. Morphology of 4nRR, 4nRG, and G fish. (A) The morphological appearance of 4nRG. (B) The morphological appearance of 4nRR. (C) The morphological appearance of G. Bar in A–C, 2 cm.

for 10 min, and observed under a light microscope to assess sperm head and tail morphology. Additional samples were dehydrated through a graded ethanol series (50 %–100 %), freeze-dried at −40 °C for 2 h (VD-250R, Japan), gold-sputtered at 15 mA for 1 min, and then examined using a JSM-6360 scanning electron microscope (JEOL, Japan) to determine sperm head diameter.

2.7. Spermatozoa motility

To evaluate spermatozoa motility, a drop of sperm was placed in the center of a marker counting chamber and activated by adding three times the volume of distilled water containing 0.1 % BSA to prevent sperm aggregation. Sperm movement was captured on video from the moment of final dilution until all movement ceased. Various motility parameters, including curvilinear velocity (VCL, μm sec^{-1}), average path velocity (VAP, μm sec^{-1}), straight line velocity (VSL, μm sec^{-1}), and the percentage of motile sperm (MOT, %), were automatically calculated during three successive 15-s tracking periods. Spermatozoa motility and kinematic parameters were evaluated using the CASA II system (Hamilton-Thorne Research, Beverly, USA) and the Animal Motility Software Manual Version 1.4.

Table 2
Morphological assessment of 4nRR and 4nRG fish[1].

Fish type	Lateral scales	Upper lateral scales	Lower Lateral scales	Dorsal fins	Abdominal fins	Anal fins
4nRG	32.2 ± 0.95	5.1 ± 0.86	6.9 ± 0.78	III + 16 ± 0.80	9 ± 0	III + 6 ± 0
4nRR	31.6 ± 0.80	5.4 ± 0.49	7.2 ± 0.40	III + 16 ± 0.63	9 ± 0	III + 6 ± 0

[1] Values were reported as mean ± SEM (n = 10), none of the traits measured significantly differed between 4nRR and 4nRG ($p > 0.05$).

Table 3
The ratios of measurable traits of 4nRR and 4nRG fish[1].

Fish type	WL/BL	BL/BH	BL/HL	HL/HH	CPL/CPH
4nRG	1.19 ± 0.03	2.68 ± 0.04	3.40 ± 0.08	1.29 ± 0.04	1.16 ± 0.08
4nRR	1.21 ± 0.01	2.64 ± 0.12	3.40 ± 0.10	1.30 ± 0.05	1.20 ± 0.09

[1] Values were reported as mean ± SEM (n = 10), none of the traits measured significantly differed between 4nRR and 4nRG ($p > 0.05$).

2.8. Statistical analysis

Data were expressed as mean ± standard error (SEM). Statistical analyses were conducted using SPSS 20.0 after verifying data normality and variance homogeneity. One-way ANOVA followed by Duncan's multiple range test was used to assess differences among groups, and significance was accepted at $p < 0.05$.

3. Results

3.1. Experimental fish breeding and hybrid generation

The crossing procedure is outlined in Fig. 1. The procedure for the preparation of 4nRR and G line has been described in our previous studies. The 4nRG individuals were produced by hybridization of G × autotetraploid *Carassius auratus*, as the G line individuals possess the ability to produce unreduced eggs. The fertilization, hatching, and survival rates for both 4nRG and 4nRR individuals are shown in Table 1.

3.2. Morphological traits

The appearance traits of 4nRG (Fig. 2A), 4nRR (Fig. 2B) and G (Fig. 2C) are shown in Fig. 2. Both 4nRG and 4nRR individuals exhibited a green-brown coloration. The counts of lateral line scales, upper and lower lateral line scales, dorsal fin rays, anal fin rays, and abdominal fin

Table 4
Mean DNA content of RCC, 4nRG, 4nRR, and G fish.

Fish type	Mean DNA content	Ratio	
		Observed	Expected
RCC	99.41		
4nRG	197.67	4nRG/2 RCC = 0.99	1
4nRR	196.72	4nRR/2 RCC = 0.98	1
G	100.46	G/ RCC = 1.01	1

Table 5
Examination of chromosome numbers in 4nRR, 4nRG, and G_3, G_4, G_5, and G_6 fish.

Fish type	No. of metaphase	Distribution of chromosome number			
		<100	100	<200	200
4nRG	200			33	167
4nRR	200			37	163
G_3	200	12	188		
G_4	200	16	184		
G_5	200	13	187		
G_6	200	15	185		

rays for both 4nRG and 4nRR are presented in Table 2. The ratios of WL/BL, BL/BH, BL/HL, HL/HH, and CPL/CPH, are provided in Table 3. No significant differences ($p > 0.05$) were observed between 4nRG and 4nRR groups for any of the countable or measurable traits.

3.3. Measurement of DNA content and examination of chromosome number

Tables 4 and 5 present the mean DNA content and the distribution of chromosome numbers for the 4nRG, 4nRR, and G line. The chromosome spreads and flow cytometry profiles of these specimens are displayed in Fig. 3A-G. As shown in Table 4 and Fig. 3A, the ploidy level of the 4nRG individuals was determined by quantifying the DNA content in caudal fin cells or blood cells, with RCC serving as the control. The DNA content of 4nRG was approximately double that of RCC ($p > 0.05$), confirming that 4nRG is a tetraploid fish. Among the analyzed samples, 83.5 % and 81.5 % of 4nRG and 4nRR metaphases contained 200 chromosomes, while 93 % of G-line metaphases contained 100 chromosomes (Fig. 3A–G, Table 5), consistent with our previous findings.

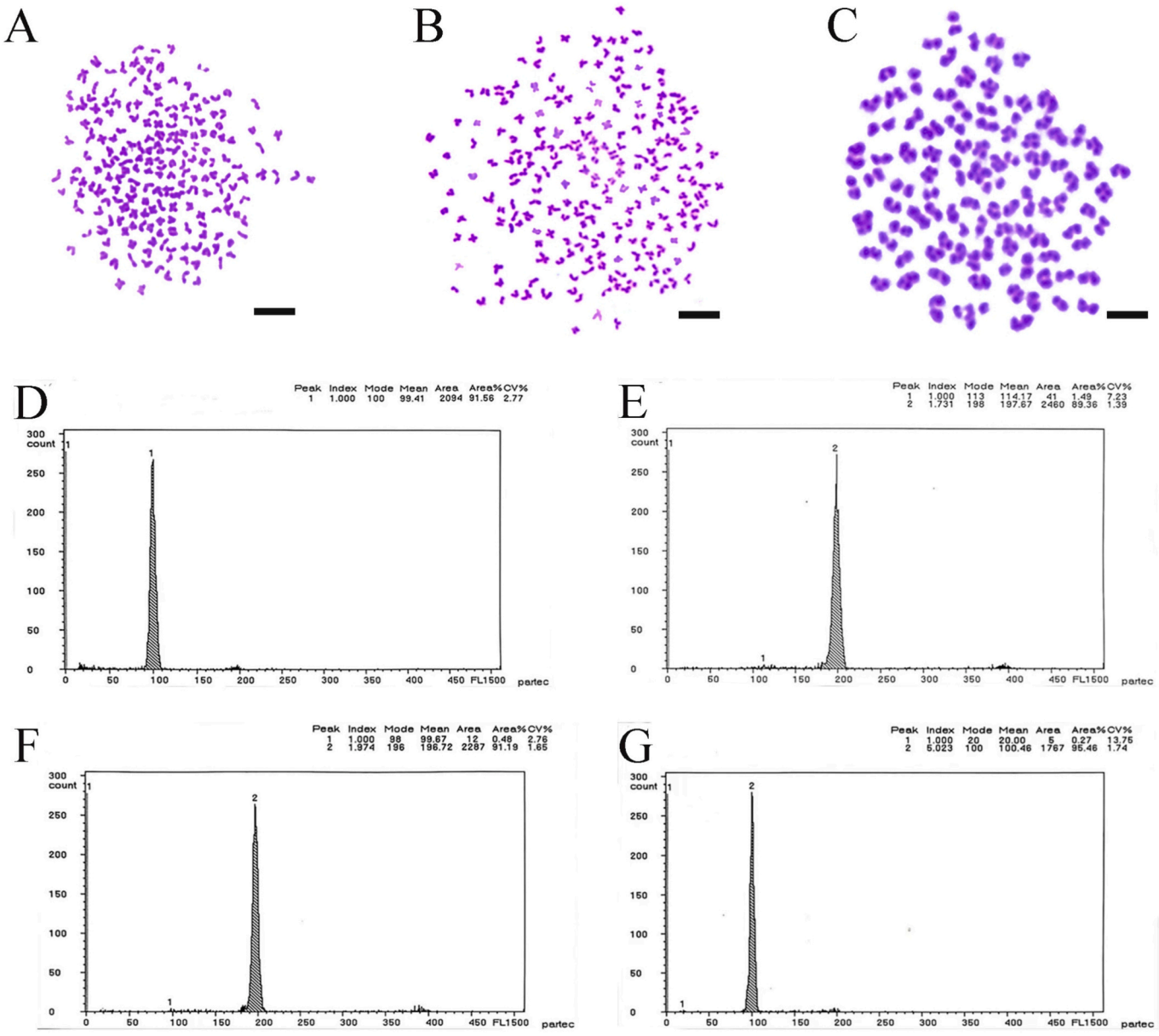

Fig. 3. Metaphase chromosome spreads and cytometric histograms of DNA fluorescence for 4nRR, 4nRG, and G fish. (A) The 200 chromosomes of 4nRG. (B) The 200 chromosomes of 4nRR. (C) The 100 chromosomes of G. Bar in A–C, 3 μm. (D) The mean DNA content of RCC (peak 1: 99.41). (E) The mean DNA content of 4nRG (peak 1: 197.67). (F) The mean DNA content of 4nRR (peak 1: 196.72). (G) The mean DNA content of G fish (peak 1: 100.46).

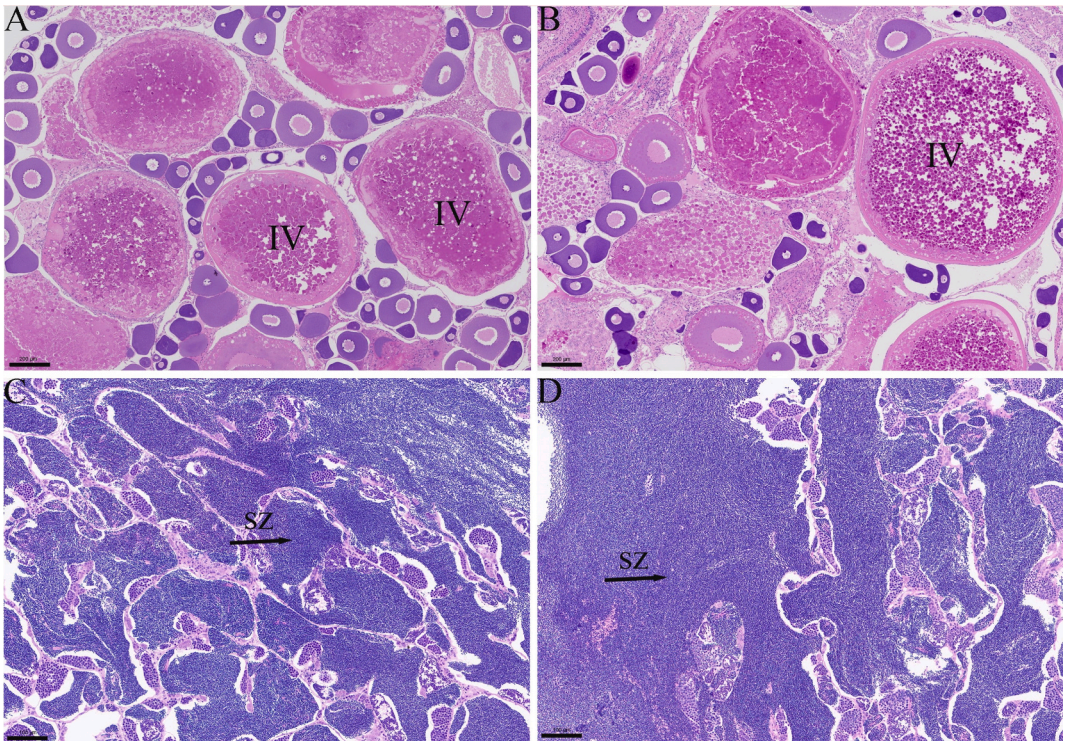

Fig. 4. Gonadal microstructure in 4nRG and 4nRR. (A) 4nRG ovary, predominantly showing phase IV oocytes with scattered phase III and II oocytes. (B) 4nRR ovary, showing normally developed oocytes in phase IV. (C) 4nRG testis contains many lobules in which there are many mature spermatozoa (black arrow) and spermatids. (D) 4nRR testis at spermatogenic phase V, with lumen filled with mature spermatozoa. Bar in A–B, 200 μm; Bar in C–D, 100 μm.

Table 6
GSI (%), relative fecundity, and sperm volume data for 4nRR and 4nRG lines[1].

Fish type	GSI(Female) (%)	GSI(male) (%)	Relative fecundity (eggs/g body weight)	Sperm volume (ml/kg)
4nRG	8.5 ± 0.23[a]	3.2 ± 0.17[a]	66 ± 4.23[a]	3.4 ± 0.16[a]
4nRR	7.1 ± 0.34	2.4 ± 0.13	50 ± 3.94	2.5 ± 0.11

[1] Values were reported as mean ± SEM (n = 10). [a] Values with different letters within the same column are significantly different ($p < 0.05$).

3.4. Gonadal and reproductive metrics

The G line fish reached sexual maturity at two years of age, whereas 4nRR and 4nRG individuals matured within one year, with both sexes of the 4nRG exhibiting fertility. Fig. 4 illustrates the microstructure of the ovaries of 4nRG and 4nRR. The ovaries of one-year-old 4nRG and 4nRR females exhibited typical development, with oocytes predominantly at stage IV (Fig. 4A, B). Similarly, the testes of 4nRG and 4nRR exhibited numerous lobules containing a considerable number of mature spermatozoa (Fig. 4C, D).

During the April breeding season, 20 individuals of both sexes from the 4nRR and 4nRG lines were sampled to measure GSI, fecundity, and semen volume, with results summarized in Table 6. Specifically, the GSI values for female and male 4nRR were 7.1 ± 0.34 and 2.4 ± 0.13, respectively, while the corresponding values for 4nRG were 8.5 ± 0.23 (female) and 3.2 ± 0.17 (male). Both 4nRR and 4nRG exhibited peak fecundity and semen volume during this period. The relative fecundity for 4nRR and 4nRG was 50 ± 3.94 eggs/g body weight and 66 ± 4.23 eggs/g body weight, respectively. Similarly, the relative semen volume was 2.5 ± 0.11 mL/kg body weight for 4nRR and 3.4 ± 0.16 mL/kg body weight for 4nRG. Analysis showed that 4nRG exhibited significantly higher GSI, fecundity, and semen volume compared to 4nRR, indicating superior reproductive capacity.

3.5. Ultrastructural and morphological features of eggs and sperm

Stereomicroscopic observation of eggs revealed that oocytes from both 4nRG and 4nRR exhibited normal morphology with a spherical shape and uniform yolk distribution. The average egg diameter was 2.88 mm for 4nRG (Fig. 5A) and 2.87 mm for 4nRR (Fig. 5B), with no significant morphological differences observed between the two groups.

Scanning electron microscopy (SEM) revealed that sperm from both 4nRG and 4nRR exhibited typical morphology, with well-defined head, midpiece, and tail structures. The sperm heads were round, with a mean diameter of 2.54 μm for 4nRG (Fig. 5C) and 2.60 μm for 4nRR (Fig. 5D).

Transmission electron microscopy (TEM) revealed that both types of sperm had normal nuclear morphology, with the nucleus occupying most of the head. The mitochondria were round or oval and were surrounded by a distinct double membrane, with a clear intermembrane space. The sperm tail originated from the basal body located in the nuclear fossa and extended through the cytoplasmic sleeve. Its axoneme exhibited the typical "9 + 2" arrangement, with 9 outer doublet microtubules surrounding a central pair (Fig. 5E, F).

3.6. Sperm motility

Sperm motility kinematic parameters of RCC, 4nRR, and 4nRG were evaluated using the CASA II system. With RCC as the reference group, the motility percentages of RCC and 4nRG spermatozoa were 92.7 ± 2.95 % and 85.2 ± 1.30 %, respectively, which were significantly higher than those of 4nRR (66.2 ± 2.65 %) (Fig. 6A). Moreover, several kinematic parameters, including straight-line velocity (VSL), curvilinear velocity (VCL), and average path velocity (VAP), were markedly higher in RCC and 4nRG compared to 4nRR, while no significant differences were observed between RCC and 4nRG (Fig. 6B–D).

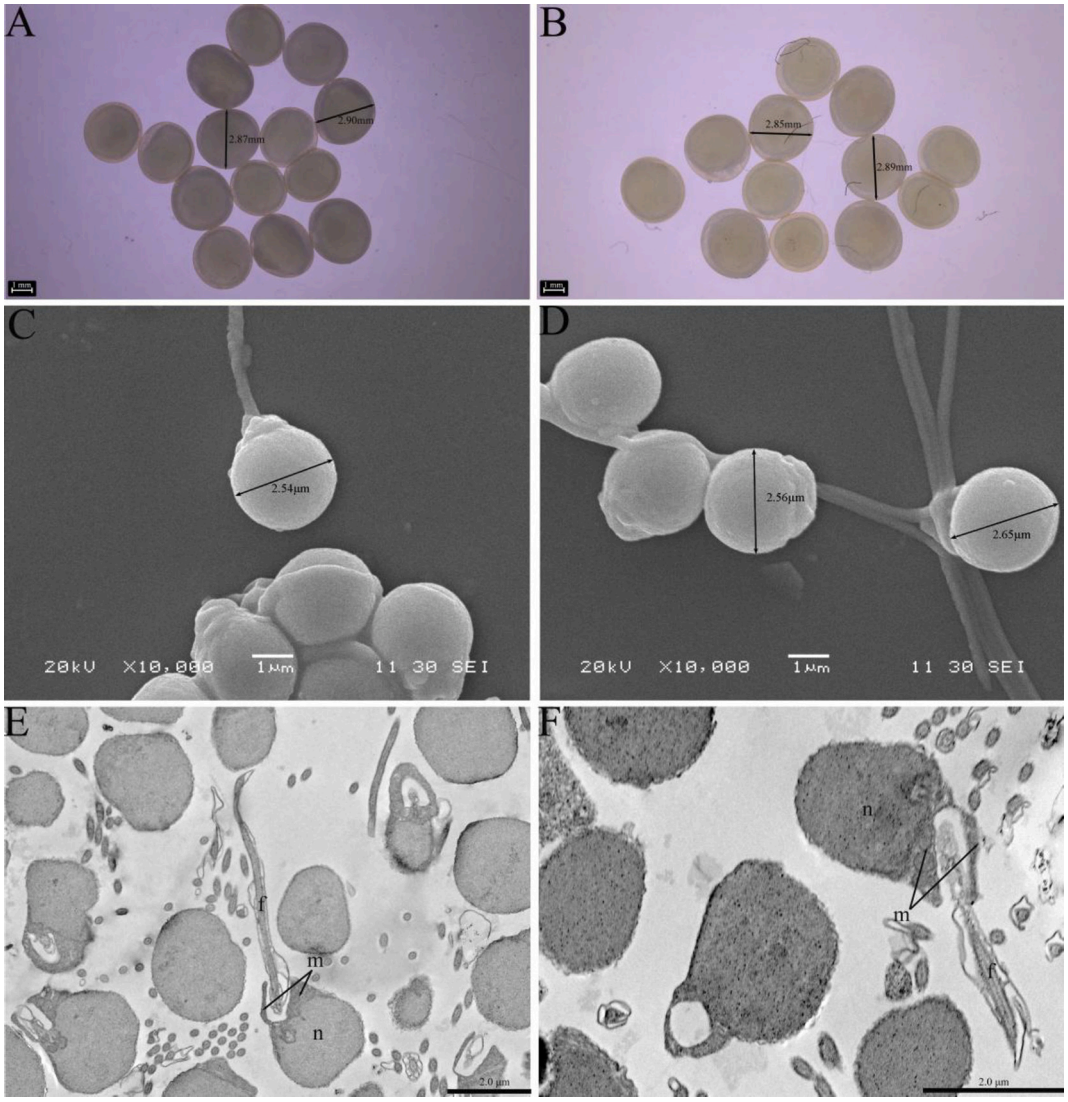

Fig. 5. Morphological and ultrastructural comparison of eggs, spermatozoa, and testes of 4nRG and 4nRR based on stereomicroscopy, SEM, and TEM. (A) eggs of 4nRG fish. (B) eggs of 4nRR fish. (C) spermatozoa of 4nRG fish. (D) spermatozoa of 4nRR fish. (E) spermatozoa of 4nRG fish. (F) spermatozoa of 4nRR fish. The diameter of sperm is indicated in the pictures. The magnification and scale bar are labeled in the pictures. n, nucleus; m, mitochondria; f, flagellum.

4. Discussion

Distant hybridization in fish is an artificial breeding technique that involves crossing distantly related taxa, typically from different genera or species, by overcoming natural reproductive isolation mechanisms. This technique offers significant advantages, including the integration of superior parental traits, generation of novel germplasm resources, and enhanced growth performance, stress resistance, and genetic diversity through polyploidization induction (Liu, 2010; Liu et al., 2022; Wang et al., 2019; Zhang et al., 2014). In previous research, an autotetraploid fish lineage (4nRR, F_2–F_{20}) was successfully established (Qin et al., 2014b). Meanwhile, gynogenesis plays a pivotal role in fish breeding by enabling sex control, monosex reproduction, and the establishment of genetically pure lines (Liu et al., 2007a, 2007b; Sun et al., 2006). In the present study, we established a diploid gynogenetic clonal hybrid line (designated the G line), which attained sexual maturity at two years of age, and was propagated to the sixth generation. The achievement of such diploid gynogenetic clonal lines holds substantial biological importance, validating that diploid eggs generate stable diploid clonal populations via gynogenesis. Moreover, these diploid eggs serve as a viable source of gametes to produce tetraploids and triploids. Using haploid gametes from diploid fish for gynogenesis requires chromosome doubling, a process that is often detrimental. Consequently, the survival rate of offspring from this process is extremely low. In contrast, a significant advantage of using diploid gametes for gynogenesis is the elimination of the chromosome doubling step, which simplifies the technical procedure and markedly improves the survival rate of offspring derived from both processes (Cherfas et al., 1994; Liu et al., 2007a, 2007b).

The combination of distant hybridization and gynogenesis technologies effectively prevents inbreeding-induced genetic degradation in fish by enhancing offspring vitality, reproductive capacity, and stress resistance through the modification of genetic material (Liu et al., 2022). Among these, artificial gynogenesis, through the homozygosity effect and heterologous sperm effect during the breeding process, allows offspring to acquire superior stress resistance compared to their parents. The heterologous sperm effect works synergistically with distant hybridization by breaking reproductive isolation and integrating genetic material from different species, resulting in offspring with enhanced stress tolerance and growth potential (Mao et al., 2020; Mao et al., 2019). Using multi-stage breeding techniques, gynogenetic individuals are backcrossed with parental stock, yielding large-scale backcross

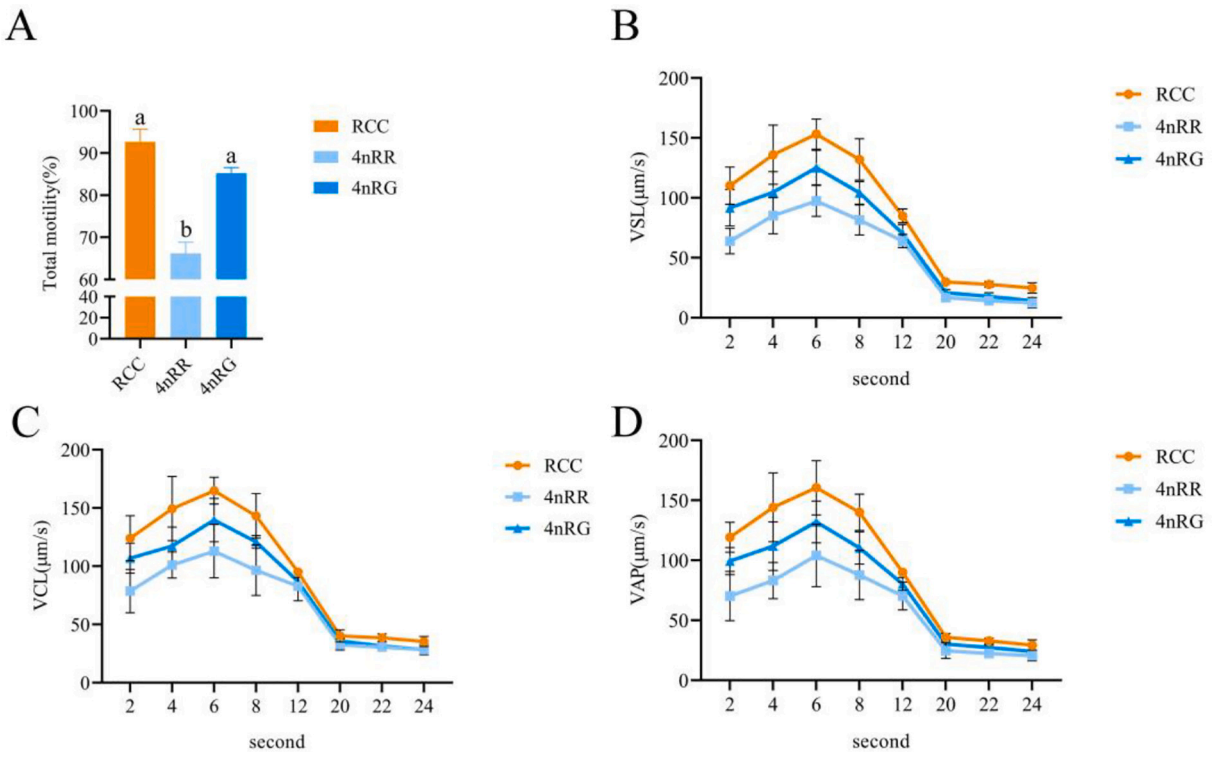

Fig. 6. Comparison of sperm motility on RCC, 4nRR and 4nRG. (A) The percentage of motile spermatozoa; (B–D) The parameters of sperm motility, including VSL (straight line velocity, μm/s), VCL (curvilinear velocity, μm/s) and VAP (average path velocity, μm/s). $n = 6$ biological replicates per group. The data are shown as the mean ± SE, and different lowercase letters indicate significant differences ($p < 0.05$).

progeny with desirable traits (Liu et al., 2025). This method has been comprehensively validated in the development of disease-resistant grass carp and improved crucian carp (Tan et al., 2023; Wang et al., 2022; Zhang et al., 2005). In this study, UV-irradiated BSB spermatozoa were used to activate inbred 4nRR female oocytes, resulting in the formation of gynogenetic progeny in the G line. The G line was subsequently backcrossed with 4nRR males to produce an improved tetraploid line.

In comparison to 4nRR, 4nRG showed no significant differences in measurable traits, enumerated traits, or morphology. 4nRG exhibited complete inheritance of 4nRR's morphological characteristics and growth patterns. The generation of fertile male and female tetraploid fish is essential for various aquatic applications. Our reproductive characterization revealed that 4nRG displayed normal development of both testes and ovaries. Female 4nRG showed consistent oocyte diameters, suggesting the production of stable diploid oocytes, similar to the male's ability to produce normal diploid sperm (Fig. 3–4). These results collectively indicate that both female and male 4nRG individuals are reproductively competent, reaching sexual maturity at one year of age and having the potential to produce offspring of both sexes. More importantly, 4nRG exhibited significant improvements in reproductive performance compared to 4nRR. The GSI, relative fecundity, relative sperm volume, and sperm motility of 4nRG were significantly higher than those of 4nRR. During the subsequent maturation period, the fertilization, hatching, and survival rates of 4nRG self-crossed progeny showed a notable increase compared to those of 4nRR.

In summary, this study successfully established a diploid gynogenetic clonal hybrid line (G line) and an improved autotetraploid strain (4nRG), and systematically analyzed the morphological traits, ploidy level, and reproductive biology of 4nRG. Both the G line and 4nRG are capable of producing stable diploid gametes, providing valuable genetic resources for polyploid breeding and offering a solid foundation for future applications in fish genetic improvement.

CRediT authorship contribution statement

Yue Zhou: Writing – original draft, Visualization, Supervision, Software, Investigation, Formal analysis, Data curation. **Ling Xiong:** Visualization, Investigation, Formal analysis. **Xu Huang:** Writing – review & editing, Methodology, Formal analysis. **Xi Dan Xu:** Visualization, Investigation, Formal analysis. **Chongqing Wang:** Writing – review & editing, Project administration. **Xiaowei Xu:** Investigation. **Kun Zhang:** Formal analysis. **Jinhai Bai:** Investigation, Formal analysis. **Zhengkun Liu:** Formal analysis, Data curation. **Yuchen Jiang:** Investigation. **Wanjing Peng:** Investigation, Formal analysis. **Enkui Hu:** Visualization, Investigation. **Siyang Li:** Visualization, Investigation. **Qian Xiao:** Investigation. **Zeyang Li:** Investigation. **Yuhan Yang:** Investigation. **Qinbo Qin:** Writing – review & editing, Project administration, Methodology, Funding acquisition, Conceptualization. **Shaojun Liu:** Writing – review & editing, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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