

Formation and characterization of artificial gynogenetic northern snakehead (*Channa argus*) induced by inactivated sperm of mandarin fish (*Siniperca chuatsi*)

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ABSTRACT

Artificial gynogenesis is a pivotal technique in the realm of aquaculture breeding. Northern snakehead (*Channa argus*) is confronting a critical issue of germplasm degradation. To solve this problem, we produced viable northern snakehead offspring via gynogenesis using UV-irradiated sperm from mandarin fish (*Siniperca chuatsi*). As expected, all gynogenetic northern snakeheads were female, as verified by sex-specific markers. To investigate the characterization of the gynogenetic northern snakehead, including whether the mandarin fish DNA fragments integrate into gynogenetic northern snakehead, we compared their morphological features, growth characteristics, DNA contents, chromosomal numbers, karyotypes, mitochondrial DNA sequences, and microsatellite DNA sequences. The gynogenetic northern snakehead exhibited dominant maternal patterns regarding morphological features, DNA contents, chromosomal numbers, karyotypes, and mitochondrial DNA traits. However, microsatellite assessments confirmed the successful integration of mandarin fish DNA fragments into the gynogenetic northern snakehead genome. Notably, the gynogenetic northern snakehead displayed superior growth compared to the maternal. We induced spawning in the 12-month-old gynogenetic northern snakehead and successfully obtained offspring. This study holds significant commercial potential for northern snakeheads and offers valuable insights for breeding.

1. Introduction

Artificial gynogenesis is a form of asexual reproduction in which eggs develop into embryos without sperm entering the egg. Artificial gynogenesis is a technique to regulate oocyte development, thus preventing the release of the dipolar body and inhibiting early division (Streisinger et al., 1981; Walker et al., 2009). Artificial gynogenesis is a valuable breeding tool in genetic breeding, with great potential for fish reproduction and genetic improvement. This technique allows for the rapid establishment of homozygous lineages, shortening the breeding cycle and improving efficiency significantly (Wang et al., 2022; Xu et al., 2015). This method has been crucial to the rapid establishment of homogenous fish strains, enhancing specific traits, and managing monosex populations, with successful implementations reported in species like grass carp (Mao et al., 2020), mandarin fish (MD) (Wu et al., 2023),

European grayling (Rożyński et al., 2023), and flatfish (Fan et al., 2016). The occurrence of paternal traits in gynogenetic fish is known as the “allo-sperm effect” (Wang et al., 1997; Yan et al., 2005; Yunjie et al., 1997; Zhang et al., 2019), underscoring its critical role in exploring DNA interactions and cross-species genetic contributions. However, no studies have focused on gynogenesis in the northern snakehead (NS).

The NS belongs to the family *Channidae* within the Order *Perciformes* and is valued for its minimal bone structure and succulent flesh, widely known as “blackfish” or simply “snakehead” (Xu et al., 2017). In response to increasing consumer preference, market demand for NS has increased, underlining its importance as a commercially valuable fish species in China (Ou et al., 2017). The dominant culture strain in China is currently a hybrid between northern snakehead (*Channa argus*) and blotched snakehead (*Channa maculata*), known for its adaptability in aquaculture systems (Ou et al., 2018). Disease problems and germplasm

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degradation during the breeding of NS-related strains, and varying quality of fry have seriously constrained the development of the NS market (Guo et al., 2022), with production reaching 553,196 tons in 2022 (China's Ministry of Agriculture., 2023). Advances in the selective breeding of fast-growing and resilient NS strains through genetic improvement will have huge market potential. Current research predominantly focuses on NS hybridization, generally within or between genera. A parallel line of research is the production of all-male NS populations through sex reversal and using super-male (YY) fish identified by molecular markers (Zhao et al., 2021). All these research methods require good female NS as parents. Given the significant degradation of NS germplasm resources, enhancing the parental northern snakehead lines through artificial gynogenesis is essential. This approach will yield substantial practical benefits for NS research.

To show that the primary genetic material of gynogenetic offspring comes from the maternal, with minor insertion from the paternal, researchers validated the origin of the progeny by comparing their genetic characteristics with those of their parents. For example, Wang et al. found that mitochondrial genes were predominantly inherited from the mother and often employed to elucidate evolutionary links between progenitors and their descendants (Wang et al., 2020).

In addition, microsatellite DNA, with its 2 to 6 base pair core sequences known as simple sequence repeats (Hansen et al., 2001), is commonly used to detect the integration of paternal DNA fragments into the offspring.

MD and NS, belonging to the order Perciformes, share an evolutionary relationship. MD exhibits rapid growth, high-quality meat, and excellent flavor. Notably, the semen of male MDs is of superior quality and abundant, making it an ideal stimulant source during the process of gynogenetic induction. There are currently no documented cases of successful hybridization between NS and MD. Our attempts to cross these two species have not resulted in viable offspring. This study aims to induce artificial gynogenesis in NS using inactivated sperm from MD to obtain gynogenetic offspring and establish a homozygous line of NS. The NS will study in detail, including analysis of their sex, morphological characteristics, chromosomal ploidy, mitochondrial DNA, and microsatellite DNA characteristics. In addition, it will investigate whether DNA fragments from the paternal species integrate into the gynogenetic northern snakehead (GNS). A rearing experiment will determine whether GNS have superior growth rates.

2. Materials and methods

2.1. Ethics statement

Researchers strictly follow ethical guidelines. They conducted breeding experiments at 2-ha outdoor ponds in the Hunan Fish Genetics and Breeding Centre. Experimental fish inhabited under conditions of certain pH (7.0–8.5), water temperatures (22–24 °C), dissolved oxygen levels (5.0–8.0 mg/L), and sufficient nourishment. For humane handling preceding necropsy, selected fish underwent sedation using 100 mg/L MS-222 (Sigma-Aldrich et al., USA).

2.2. The GNS formation process

At the Hunan Fish Genetics and Breeding Center, three-year-old NS and MD from Dongting Lake were carefully selected, with NS averaging 46.36 ± 0.28 cm and 857.00 ± 36.05 g, and MD at 38.00 ± 1.15 cm and 1000.00 ± 90.00 g. MD sperm, after collection, was diluted with Hank's solution (1:20) and then subjected to UV radiation via ZSZ20D lamps at 254 nm, maintaining an exposure of 3000 to 3600 mJ/cm² for 15–30 min. The motility of the sperm was assessed under a microscope after treatment. Irradiation was halted when motility reached 30% of the initial value, and samples were subsequently stored at 4 °C in opaque dark glass tubes to prevent light-induced degradation. Ova induction utilized mature NS females, subjected to a regimen starting with an

injection of 4 µg/kg LH-RH analog, followed 12 h later by a combined injection of 16 µg/kg LH-RH analog and 700 IU/kg HCG after 12 h (Ou et al., 2018). After 18–22 h, NS eggs were harvested and collected in sterile containers. UV-treated spermatozoa were mixed with collected eggs using feather stirrers. Later, eggs underwent a 15–30-min cold shock in 4–6 °C water. Upon Post-cold treatment, fertilized eggs were incubated in 0.5 m diameter containers for incubation at 25–28 °C. Embryos were then placed in $3.5 \times 2.5 \times 1.2$ m concrete pools. Around 2000 embryos were randomly collected to assess fertilization (number of embryos/number of gastrula stage eggs $\times 100\%$), hatching (number of hatchlings/eggs $\times 100\%$), and survival rates (number of autonomously feeding larvae/eggs $\times 100\%$).

2.3. Exploration of optimal gynogenesis conditions

For each cohort of fish eggs, our experimental design consisted of one experimental group and three control groups. The first control group involved self-crossing NS eggs with NS sperm. The second control group involved NS eggs fertilized with MD sperm, while the third control group involved crossing NS eggs with UV-irradiated MD sperm. The experimental group differed by fertilizing NS eggs with UV-irradiated MD sperm followed by a cold shock protocol. The methodology aimed to activate NS eggs with UV-treated MD sperm. Eggs and sperm were mixed and fertilized in 28 °C water for 1, 2, 3, 4, or 5 min. Eggs were then submerged for 15 min, 20 min, or 25 min in 4 °C water to achieve optimal cold shock efficacy. To ensure consistent outcomes, we replicated this experiment three times. We set a sample size of 2000 embryos within each experimental and control group to determine hatchability and early survival rates. Hatchability is the ratio of hatched larvae to total eggs, while early survival rates are the proportion of enduring larvae relative to the aggregate egg count (Liu, 1993).

2.4. Measurement of countable traits

This study randomly selected 20 three-month-old NSs, 20 three-month-old MDs, and 20 three-month-old GNSs. Examined traits included counts of dorsal fins, abdominal fins, and anal fins, along with upper lateral, lateral, and lower lateral scale counts. This study measured body metrics such as body length (BL), total length (WL), body height (BH), head length (HL), caudal peduncle length (CPL), and height (CPH). Ratios and their standard deviations for WL/BL, BL/BH, BL/HL, BL/CPL, and CPL / CPH were calculated, providing insight into the morphological variances across NS, MD, and GNS.

2.5. Growth performance of GNS

NS juveniles were used as controls to assess the growth performance of GNS. From each group, 30 individuals of 10 cm body length were selected and placed in $250 \times 250 \times 100$ cm tanks. This study raised both sets of larvae under similar conditions, consistently supplying fish for feeding. Each experimental pool housed 30 fish, forming two parallel groups for the study. At the experiment's outset, the researchers randomly selected ten fish from each group and accurately recorded their body length (± 0.5 cm) and weight (± 0.5 g) as baseline data. Sufficient fish was provided in all tanks to ensure uniform feeding conditions. From June to September 2023, ten fish from each group were selected monthly for measuring body length and weight to monitor their growth performance. To better reflect the increase in weight, the Average Daily Gain (ADG) was calculated using the following formula (Korkut et al., 2007): $ADG = (W_f - W_i) / (D_f - D_i)$, where W_f = the average final weight; W_i = the average initial weight; D_f = the final day; and D_i = the first day. During the 10-month breeding period, we assessed the fertility of the GNS by examining their gonads.

2.6. Measurement of DNA content

Ploidy levels in GNS were determined by quantifying DNA content using a flow cytometer (Cell Counter Analyzer, Partec, Germany). DNA content in red blood cells was evaluated for NS, MD, and GNS. Twenty individuals from each fish category at 3 months of age were selected for analysis. The method described in the published paper was followed (Wu et al., 2023). DNA content measurements were conducted using uniform experimental settings. The red crucian carp DNA content was used as the control for calibration (Liu et al., 2007).

2.7. Preparation of chromosome spreads

We carried out chromosome preparations of kidney samples from ten individuals of NS, MD, and GNS, each collected in March. We meticulously observed the morphology and counted the chromosomes under a microscope, analyzing 100 metaphase spreads (ten per specimen) for each fish type. This approach allowed us to determine their respective chromosome numbers and karyotypes, providing insights into their genetic structure (Wang et al., 2018).

2.8. Mitochondrial DNA sequence and phylogenetic analysis of GNS

Total genomic DNA was extracted from peripheral blood samples of ten GNS individuals utilizing standard techniques, serving as a template for further analysis (Zhang et al., 2020). Amplification of the complete mitochondrial DNA sequences from the GNS genome was achieved by using the primers previously documented (Zhang et al., 2015) and the primer list detailed in Table 6 (newly designed primers are indicated with an “*”). The sequencing data were analyzed with BioEdit software, version 7.0 (Hall et al., 2011). Comprehensive mitochondrial DNA sequences were sourced from the GenBank database, including NS (NCBI: NC_015191.1) and MD (NCBI: NC_015822.1). The structural examination of these mitochondrial sequences was facilitated by the Mitofish online platform, available at <http://mitofish.aori.u-tokyo.ac.jp/annotation/input/>.

2.9. Microsatellite DNA of GNS

The phenol-chloroform extraction method isolated Genomic DNA from the NS, MD, and GNS tail fins. The DNA's concentration and integrity were verified via agarose gel electrophoresis. For the PCR amplification of genetic loci within NS, GNS, and MD samples, 60 microsatellite primer pairs were used. Among these, only the primer pair TRANSI-2 (Forward: AGGAGACTCTGTAGAAGGACA and Reverse: GGAGAGGAGACAGTGGAGAC) fulfilled our specificity requirement (ZHANG et al., 2014). The PCR was executed following the procedures and conditions documented in prior research (Zhang et al., 2014). We ran the amplified products in 8% polyacrylamide gel electrophoresis (PAGE) and measured the size according to a pBR322 DNA/MspI ladder (Tiangen), ensuring precise genotypic characterization.

2.10. Sexual identification of GNS

Peripheral blood samples were randomly drawn from the caudal veins of 21 GNS individuals, from which genomic DNA was extracted using the Takara Min iBEST Universal Genomic DNA Extraction Kit v5.0, as described (Zhang et al., 2020). The amplification of sex-specific markers in male controls and GNS specimens utilized reported sex-specific primers (Forward: TTCAGCAATAAGCGAGACAATG, Reverse: CCGATCTGTTGGACCTGTTAG), adhering to the PCR protocols (Sun et al., 2023). The cycling conditions included a 94 °C initial denaturation for 30 s, 56 °C annealing for 30 s, and a 72 °C extension for 1 min and 30 s for 35 cycles. Electrophoresis on 2.0% agarose gel was then used to analyze the PCR products.

3. Results

3.1. GNS optimal cold shock treatment conditions

This experiment attempted to determine the optimal conditions for NS formation by fine-tuning the post-fertilization development time and cold shock time. The analysis in Table 1 shows that a post-fertilization development time of 3 min and a cold shock time of 20 min minimized malformed GNS progeny with a survival rate of up to 5%. Deviation from the optimal cold shock time resulted in increased malformations in offspring. GNS with a cold shock time of 20 min had the highest survival rate of all groups tested. A development time of 3 min after fertilization gave the best results. A developmental time of 3 min after fertilization and a cold shock time of 20 min was most effective in reducing malformed progeny while maintaining viability. Observations from the first control group indicate that the *Channa argus* eggs used in all experimental groups were mature and healthy. Results from the second and third control groups demonstrate that MD sperm, regardless of UV irradiation, could not successfully fertilize NS eggs to produce viable offspring.

3.2. Morphological features of GNS

This experiment aimed to examine the morphological characteristics of NS and its GNS compared to MD. Representative images of the morphological features are shown (Supplementary Fig. 1). Analysis of countable and measurable traits, as detailed in Table 2, showed NS and GNS shared numerous morphological features, notably in the number of abdominal and anal fins, indicating that GNS preserves NS's key morphological traits. Conversely, MD displayed substantial differences in all countable traits from NS and GNS, underscoring the inherent morphological divergence between the species.

Further, according to Table 3, NS and GNS exhibited similar body proportions, particularly in the body length to caudal peduncle length ratio (BL/CPL), which remained consistent across the species. These data imply the trait's stability through evolutionary history. These observations affirm GNS's morphological congruence with NS and its distinctiveness from MD.

Table 1

Effects of different post-fertilization development time and cold shock times on gynogenetic in GNS.

Post-fertilization development time	Cold shock time	Number of experimental eggs	Hatching rate (%)	Survival rate (%)
1 min	15 min	2000	0%	0%
	20 min		0%	0%
	25 min		0%	0%
2 min	15 min		0.15%	0.05%
	20 min		0.25%	0.05%
	25 min		0.05%	0%
3 min	15 min		2%	1.5%
	20 min		6%	5%
	25 min		1%	0.8%
4 min	15 min		0.05%	0.05%
	20 min		0.15%	0.10%
	25 min		0.05%	0.05%
5 min	15 min		0%	0%
	20 min		0%	0%
	25 min		0%	0%
First control group	0		89.15%	84.65%
Second control group	0		0%	%
Third control group	0		0%	0%

Table 2
Comparison of the countable traits in NS, MD, and GNS.

Fish type	Lateral line scales	Upper lateral line scales	Lower lateral line scales	Dorsal fins	Abdominal fins	Anal fins
NS	61–68	8–10	17–20	47–52	6	30–34
MD	112–116	31–32	53–55	XI +12–14	I + 5	III + 9–11
GNS	65–68	9–10	16–20	49–52	6	32–34

Table 3
Comparison of the measurable traits among NS, MD, and GNS.

Fish type	WL/BL	BL/BH	BL/HL	BL/CPL	CPL/CPH
NS	1.15 ± 0.01	5.68 ± 0.44	3.84 ± 0.20	15.40 ± 2.07	0.69 ± 0.10
MD	1.41 ± 0.01	2.28 ± 0.30	2.46 ± 0.20	16.15 ± 2.21	1.18 ± 0.10
GNS	1.16 ± 0.01	5.48 ± 0.50	3.84 ± 0.50	15.80 ± 2.51	0.68 ± 0.20

3.3. Growth performance of GNS

To investigate how the NS and GNS groups differed in their growth under the same rearing environment. We conducted a growth performance analysis based on the data presented in Table 4. After 3 months of feeding, the GNS group achieved an average body length of 24.7 ± 0.52 cm and an average weight of 234 ± 10.3 g. Compared to the NS group, which recorded an average body length of 23.2 ± 0.26 cm and a 202 ± 7.21 g weight. We employed the Student's *t*-test to analyze the data. After 90 days of cultivation, the GNS group showed a significant increase in body length by 6.5% and weight by 15.84% ($P < 0.05$). The ADG of NS and GNS were 2.24 g/d and 2.6 g/d throughout the 90-day breeding experiment. This significant growth differential may suggest a superior growth efficiency or a higher feed conversion ratio in GNS specimens. The data elucidate that the GNS consistently surpassed NS in growth performance during the entire period. In Fig. 1, an individual GNS aged 10 months is documented. Precise measurements indicate that this GNS has a body length of 45 cm (± 1.5 cm) and weighs 830.62 g (± 10 g). After 10 months of aquaculture, three randomly selected GNS specimens underwent anatomical testing and exhibited well-developed gonads (Fig. 1D). These data demonstrate that GNS exhibits superior growth compared to NS.

3.4. DNA content of GN, MD, and NS

This study aimed to investigate the DNA content of GNS, MD, and NS using red carp as a control group. The results, remarkably the comparison of NS (Fig. 2A) and GNS (Fig. 2D), are shown in Fig. 2. The DNA content of NS (Fig. 2A) and GNS (Fig. 2D) was 40.16 and 40.25 relative to red crucian carp. The measured data indicates that the DNA content of GNS and NS is almost identical, with a ratio of approximately 1:1. This suggests that both GNS and NS have maintained their diploid status and have a high degree of concordance regarding genetic material content.

Table 4
The growth performance data on body length and body weight from NS and GNS in 2023.

Fish type	Initial day		30 days		60 days		90 days	
	Length (cm)	Weight (g)	Length* (cm)	Weight* (g)	Length* (cm)	Weight* (g)	Length* (cm)	Weight* (g)
NS	10.3 ± 0.35	21.3 ± 0.26	17.1 ± 0.36	79 ± 3.61	21.17 ± 0.61	147 ± 8.66	23.2 ± 0.26	202 ± 7.21
GNS	10.2 ± 0.17	21.7 ± 0.36	17.7 ± 0.35	84 ± 5	22.9 ± 0.6	188 ± 8.54	24.7 ± 0.52	234 ± 10.39
GNS – NS	– 0.1	0.4	0.6	5	1.73	41	1.5	32

3.5. Chromosome numbers and karyotypes

This experiment examined the chromosome number and karyotype of GNS, NS, and MD. We selected 100 samples each from mid-stage cells of NS, MD, and GNS for chromosome counting and karyotype analysis to gain insight into the similarity between GNS and NS regarding chromosome number and karyotype structure-specific results presented in Table 5 and Fig. 2. The study found that 93% of mid-stage NS cells had a karyotype of 48 chromosomes with a structural configuration of 4SM + 20ST + 24 T (Fig. 2B-C). Similarly, 92% of mid-stage GNS cells had the same karyotype structure of 48 chromosomes, i.e., 4SM + 20ST + 24 T (Fig. 2E-F). In contrast, 89% of MD samples had 48 chromosomes with a karyotype formula of 6SM + 12ST + 30 T (Fig. 2H-I).

3.6. Mitochondrial DNA sequence and phylogenetic analysis of GNS

To verify the homology of the mitochondrial DNA of GNS with that of MD and NS, we amplified the mitochondrial DNA of GNS. After sequencing and splicing, we obtained a full-length 16,558 bp mitochondrial DNA sequence of GNS. In comparison, MD's full-length mitochondrial DNA (GenBank accession number: JF972568.1) was 16,496 bp, while NS's full-length mitochondrial DNA (GenBank accession number: NC_015191.1) was identical to that of GNS, which was 16,558 bp. The arrangement of the related genes is shown in Fig. 3. Using the sequence of the ND5 gene in mitochondria as an example, in the amplified DNA sequence, we observed very few base variants in the amplified DNA sequence. We found no deletions or insertions of bases or fragments among NS and three GNSs (Fig. 4). Further analysis showed that GNS's mitochondrial DNA sequences were highly similar to NS's, with a homology of 99.8%.

3.7. Microsatellite DNA of GNS

This study aimed to investigate the pattern of microsatellite DNA in MD, NS, and GNS by PCR amplifying specific fragments using TRANSI-2 primers. The results are presented in Fig. 5, which shows that five significant fragments identical to GNS were identified in the NS sample, indicating a high degree of homology between GNS and NS. Only one homologous fragment matching GNS was identified in the MD sample, indicating a relatively low genetic linkage between GNS and MD. Notably, GNS exhibited specific bands matching MD at 216 bp and 204 bp, which were not observed in the NS samples. This phenomenon suggests the possible insertion of gene fragments from MD in GNS. We substantiated this hypothesis through recovery sequencing of fragments in GNS, NS, and MD (Fig. 6).

3.8. Sexual identification of GNS

The study used specific sex markers to confirm GNS sex. Agarose gel electrophoresis analysis of the samples showed that a distinct band at approximately 1200 bp appeared in the male control samples. In contrast, this band did not appear in any of the randomly selected GNS samples (Fig. 7), indicating that all of these GNS samples were female.

3.9. Breeding strategy for gynogenesis

To verify the reproductive capability of GNS, our research team

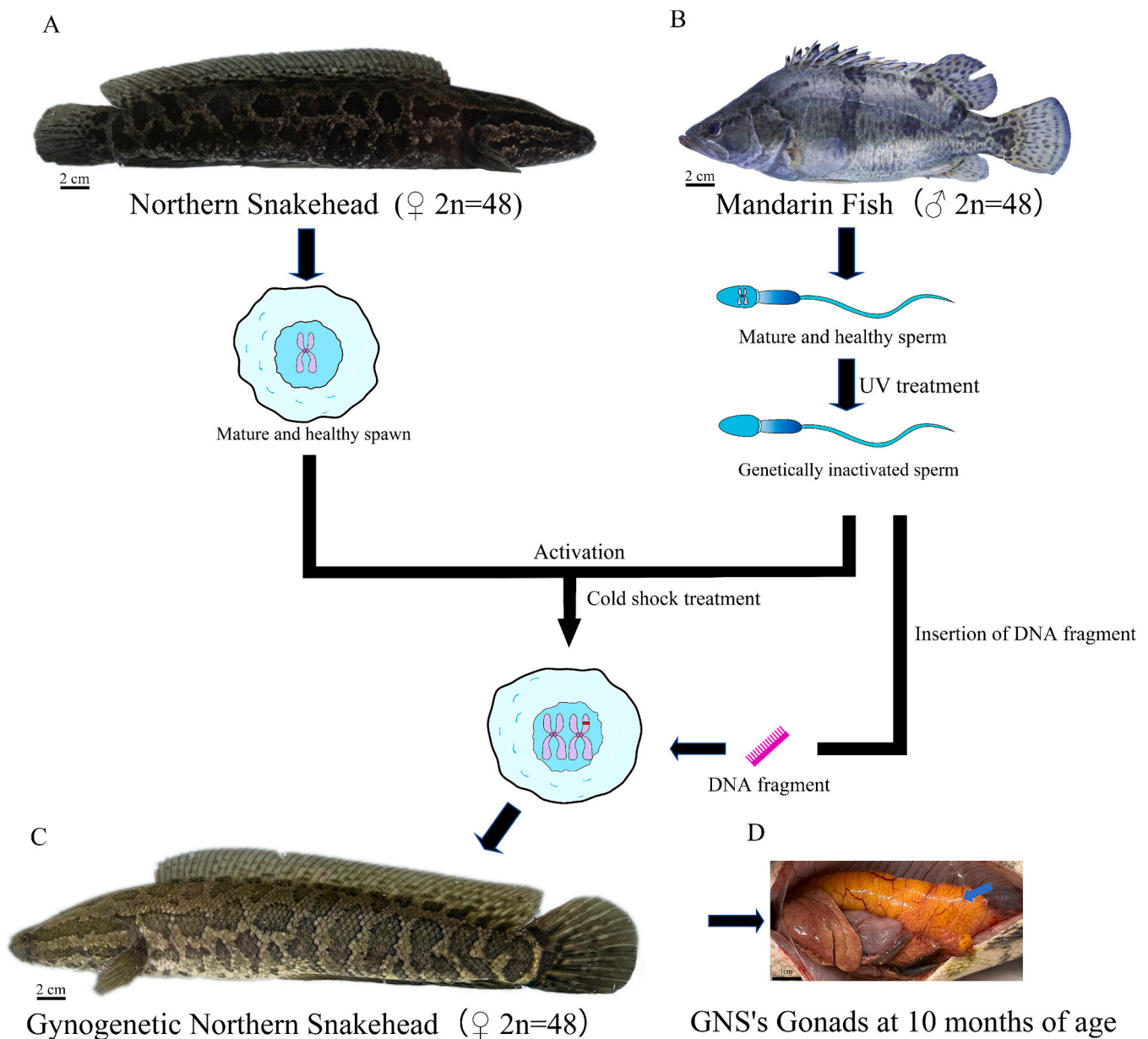


Fig. 1. Formation procedure and appearance of NS, MD, and GNS. The appearance of NS (A). The appearance of MD (B). The appearance of GNS (C). The GNS gonads were well developed after 10 months of aquaculture, as shown by the blue arrows (D). In May 2024, we catalyzed a 12-month-old GNS and obtained a viable backcross progeny population. In subsequent breeding, we will continue to self-cross this backcross population to expand further. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

successfully induced spawning in GNS on May 15, 2024 and obtained living offspring. The experiment results demonstrate that GNS can usually pair with male NS and exhibit good fertility. Although GNS has advantages over NS, their limited numbers make direct market promotion challenging. Therefore, our team developed a novel breeding strategy, as shown in Fig. 8, to maximize the transmission of gynogenesis advantages through reproduction. Initially, we used a backcross strategy between gynogenetic females and typical males to produce the F_1 generation. From the F_1 population, we can select superior male individuals and cross them with gynogenetic females to produce the G_2 generation. The F_1 and G_2 populations may retain the “allo-sperm effect,” which may result in the advantages in these populations. F_1 and G_2 populations will be able to expand through self-crossing. As shown by the blue dashed lines in Fig. 8, these high-quality populations serve as the foundational parent populations for preparing the strains of the strains derived from gynogenesis, which meet market demands while

exhibiting desirable traits. Through this strategy, we can maximize the inheritance of advantageous characteristics resulting from the “allo-sperm effect” in artificial gynogenesis. This approach allows us to improve the strains derived from gynogenesis while meeting quantitative demands. Our team has successfully established the F_1 population and will continue to expand the existing populations. We will continue to build F_n , G_1 , and G_n populations. This breeding strategy increases populations with the “allo-sperm effect” and some advantages, and it solves the problem of the limited numbers of gynogenetic fish in the market. This novel breeding strategy is also suitable for GNS.

4. Discussion

We put forward the concept of macro-hybrid and micro-hybrid, drawing on extensive experimental results from fish-distant hybridization and gynogenesis made by our team. The term “macro-hybrid” refers

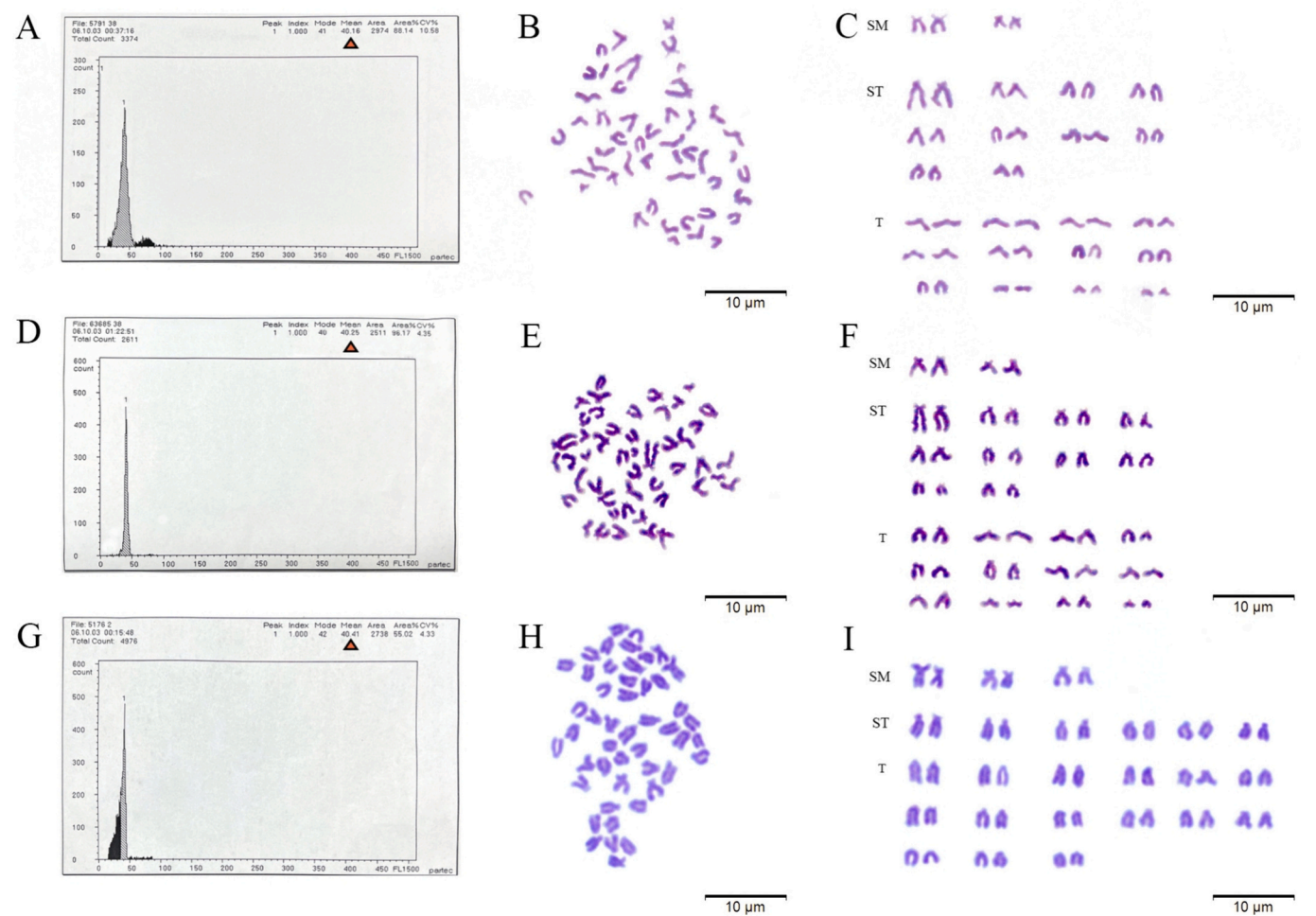


Fig. 2. Chromosomal traits of NS, GNS, and MD. The chromosomal numbers and karyotype of NS (B–C). The chromosomal numbers and karyotype of GNS (E–F). The chromosomal numbers and karyotype of MD (H–I). Cytometric histograms of DNA fluorescence for NS (A), GNS (D), and MD (G).

Table 5
Chromosome numbers in NS, MD, and GNS.

Fish type	Number of metaphases photographs	Distribution of chromosome number				
		<46	46	47	48	>48
NS	100	3	1	2	93	1
MD	100	4	2	3	89	2
GNS	100	2	1	4	92	1

Table 6
Mitochondrial full-length amplification primer sequence of GNS.

Primers	Forward primer sequences (5'-3')	Reverse primer sequences (5'-3')
Mt1	AAGCCTCGCCTGTTACCAA	TCTGGGAAAATGGGGATGTG
Mt2	ATTCGCCCTGTTCTACCTGG	CAGGGACTGGGAGGTTTCA
Mt3	CCTCACACCTGCCACAATC	TGGATTTTGTTCGAAGTCTG
Mt4	CCTCAGCGGCATTGTCTTAG	AAGGGTGGAAGTCCGGTTGT
Mt5	CCTTCCCTGAGTTCTCTTCC	CAAGGGAGTGGGAGCAGTAG
Mt6	GCTCAGCCCGACTCCCCTTT	GGCGGCTGTGATAAGGGTGC
Mt9	CCTGTACTACGGCTCCTACC	GGGGTGCTTAGTGTGTTAG
Mt10	GCATTTTAGGTTATCAAGAGCA	GGGCTTACTGGCTAAATCAT
Mt11	CCCTTACACCGAGAAGTCAT	GGCTGCTCTGGTGTCTAAAG
Mt7*	TGAGTCATCATCATCCCTAT	TGATTCGACACCCCTCC
Mt7–8*	TTATCGGCTGGGAAGGTGTC	GGGCTCTTCGGGCAGTTT
Mt8*	CTAGACTGCCGAAGAGC	GGAGGACATAGCCAACGA

to offspring from distant hybridization that possess two distinct

subgenomes, each inherited from one of the two parental species, such as allodiploid and allotetraploid lineages (Liu et al., 2004; Wang et al., 2014; Wang et al., 2020). The concept of “micro-hybrid” refers to offspring, including autodiploid and autotetraploid lineages, and those resulting from artificial gynogenesis, whose genome almost originates solely from the maternal parent but in which specific DNA fragments derived from the paternal parent insert (Gong et al., 2019; Mao et al., 2020; Wu et al., 2023). In this context, the GNS is a typical micro-hybrid example. Gynogenetic fish are usually female and fertile but might exhibit paternal-specific genotypes due to the “allo-sperm effect.”

We developed a gynogenetic breeding strategy (Fig. 8) to sustain the offspring of artificial gynogenesis. In this approach, female gynogenetic fish are mated with males of regular fish (backcrossing), resulting in F₁ generation, which may carry the original paternal-specific gynogenetic DNA fragments due to the “allo-sperm effect.” The males of the F₁ generation are mated with the gynogenetic females (G₁) to produce the new generation (G₂). The males and females of the F₁ generation are mated to produce another new generation (F₂). The populations (G₂ and F₂) may also present the “allo-sperm effect,” which can produce phenotype variations. With this breeding strategy, both males and females of the individuals with the “allo-sperm effect” are formed, and the populations with the “allo-sperm effect” may be stably inherited.

This strategy has been or will be used in these combinations in many combinations, including gynogenetic grass carp (Mao et al., 2020), gynogenetic mandarin fish (Wu et al., 2023), and gynogenetic largemouth bass (Zhong et al., 2024), which are mated with normal males, respectively. For example, we mate female gynogenetic grass carp (Mao et al.,

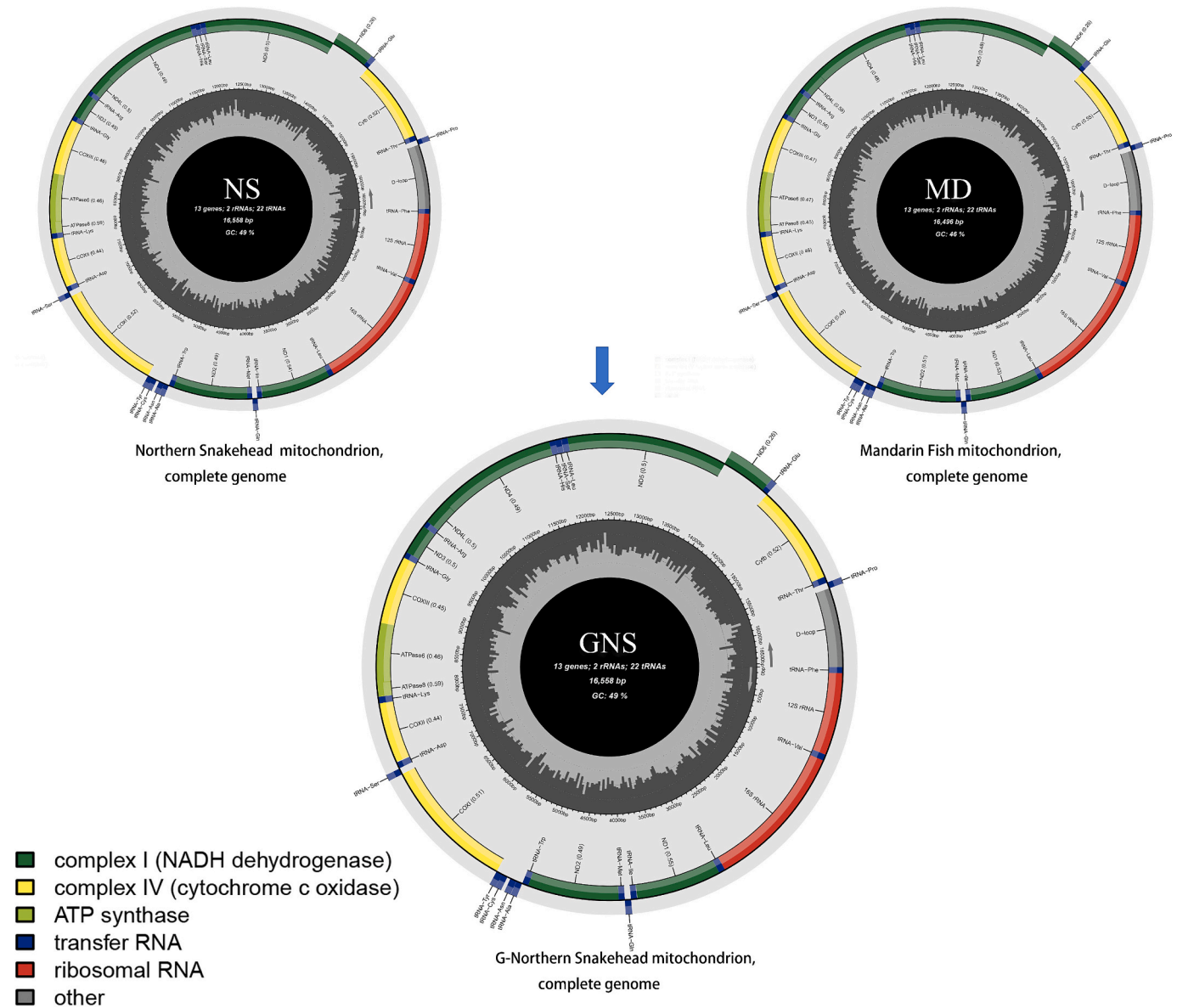


Fig. 3. Complete mitochondrial structures of NS (16,558 bp), MD (16,496 bp), and GNS (16,558 bp).

2020; Mao et al., 2019) with male grass carp to produce the F₁ generation, including both males and females, which also shows the “allo-sperm effect” and the disease-resistant ability. Therefore, this fertile bisexual F₁ group would be available as a new genetic resource for grass carp to produce more disease-resistant grass carp. This breeding approach is essential in production applications. Artificial gynogenesis has been used in breeding many fish species, and some have shown excellent traits (Gong et al., 2019; Mao et al., 2020; Wu et al., 2023).

The genome of the artificial gynogenetic fish will be randomly integrated with gynogenetic DNA fragments from the patrilineal, as shown in Fig. 1. When the artificial gynogenetic fish genome successfully integrates these gynogenetic DNA fragments, they may affect the traits of the artificial gynogenetic fish, including growth rate, stress tolerance, body colour, etc. The GNS has a growth advantage, as shown in this paper. As shown in this paper, the GNS has a growth advantage, but it is worth continuing to investigate whether the GNS has other benefits. Artificial gynogenesis is equivalent to pre-screening the progeny population. Therefore, the number of individuals produced is insufficient to meet the market demand. Our team has innovatively proposed a reproductive strategy for snakehead's artificial gynogenetic

development, as shown in Fig. 8. Through our team's long experience in breeding, we developed an approach to select males from GNS backcross progeny for crossing with GNS. Our team has completed the establishment of the F₁-NS population in 2024, and we will expand the population by self-crossing F₁-NS. At the same time, we will generate a G₂-NS population by crossing. This strategy ensures that the good traits of GNS can be inherited as much as possible, and at the same time, it solves the problem of an insufficient number of males in the market. Such a breeding strategy can provide a good breeding strategy for promoting many excellent artificial gynogenetic fishes.

To improve the germplasm resources of NS, we initiated the present study. In this study, we effectively employ MD sperm to trigger the initiation of the gynogenesis in NS, resulting in the generation of surviving offspring. We explored the ideal conditions for GNS production by optimizing the induction in a dose-dependent manner. Gynogenesis facilitates the accurate determination of the sex, making it suitable for breeding single-sex populations, such as all-female or all-male species, to meet specific breeding needs (Cheng et al., 2019; Manan et al., 2022; Rożyński et al., 2023). We confirmed this in the present study by sex-specific molecular markers of NS. GNS is an all-female community.



Fig. 4. Nucleotide sequence alignment of the mitochondrial ND5 gene for NS, MD, and the three GNSs (GNS1–3). (The yellow background represents bases where GNS differs from NS, and the dots represent bases identical to NS.) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Examination of the GNS gonads showed excellent reproductive performance. Gynogenesis with the “allo-sperm effect” increases the genetic variability and survival of the population, thereby enhancing the financial gains of aquaculture (Dapper and Wade, 2016). DNA fragments from MD were identified in GNS using targeted primers in this study, probably leading to GNS's enhanced growth over NS, as evidenced by

cultivation trials. The GNS was confirmed to be a product of artificial gynogenesis. It was supported by thoroughly evaluating their morphological attributes, DNA composition, chromosome count and structure, mitochondrial DNA, and microsatellite DNA analysis.

Our study has found that the sperm from the MD is remarkably effective at initiating gynogenesis in the NS. Adjusting the post-

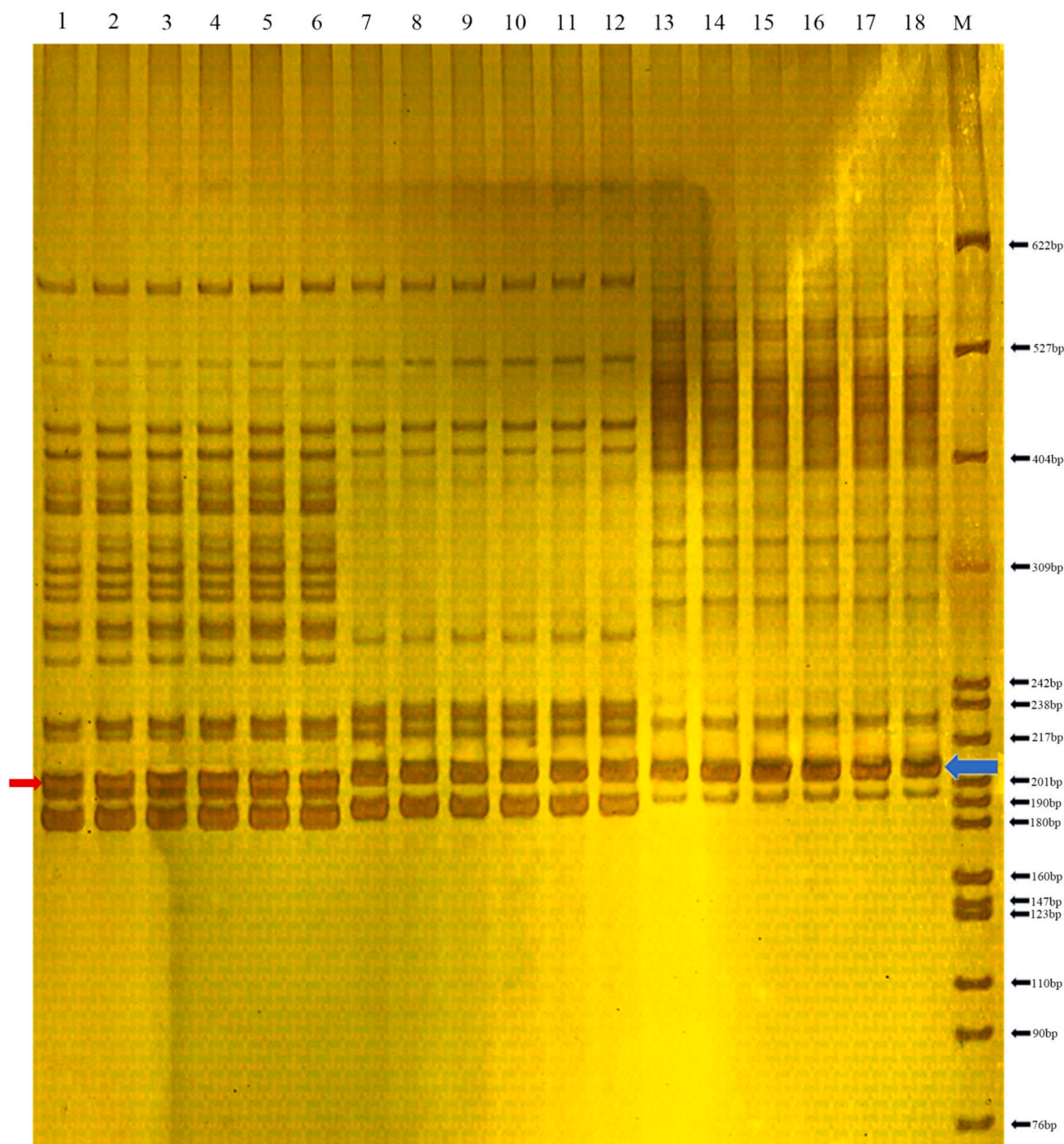


Fig. 5. Microsatellite patterns of NS (No. 1–6), GNS (No. 7–12), and MD (No. 13–18) fish. Electropherogram of microsatellite DNA patterns produced by the primer pair in NS, GNS, and MD. Lanes 1–6 represent NS, 7–12 represent GNS, and 13–18 represent MD. The blue arrow indicates the DNA bands derived from MD. The red arrow indicates the DNA bands from NS that are most similar to those shown by the blue arrow. M represents the pBR322 DNA/MspI marker. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

fertilization development time and the length of the cold shock has allowed us to create ideal conditions that reduce the incidence of deformities in offspring and increase the survival rates of GNS. Remarkably, the protocol involving 3 minutes for post-fertilization development time and a subsequent cold shock lasting between 20 min proved to be the most successful approach, highlighting the importance of accurately adjusting these parameters. Our research observations indicate that in the formation process of GNS, conditions are both analogous to and significantly distinct from previous study outcomes compared to

existing research literature (Chen et al., 2009; Manan et al., 2022). During the study exploring artificial gynogenesis in NS, the researchers summarized three critical factors for the success of artificial gynogenesis based on the experience gained. Initially, selecting heterologous sperm necessitates a balanced approach, avoiding genetic material either excessively akin to or divergent from the target fish (Cramer et al., 2016). Secondly, in the intermediate stage, before post-fertilization and cold shock treatment, it is imperative to meticulously manage the timing to avoid failure to capture the critical juncture at which the egg expels

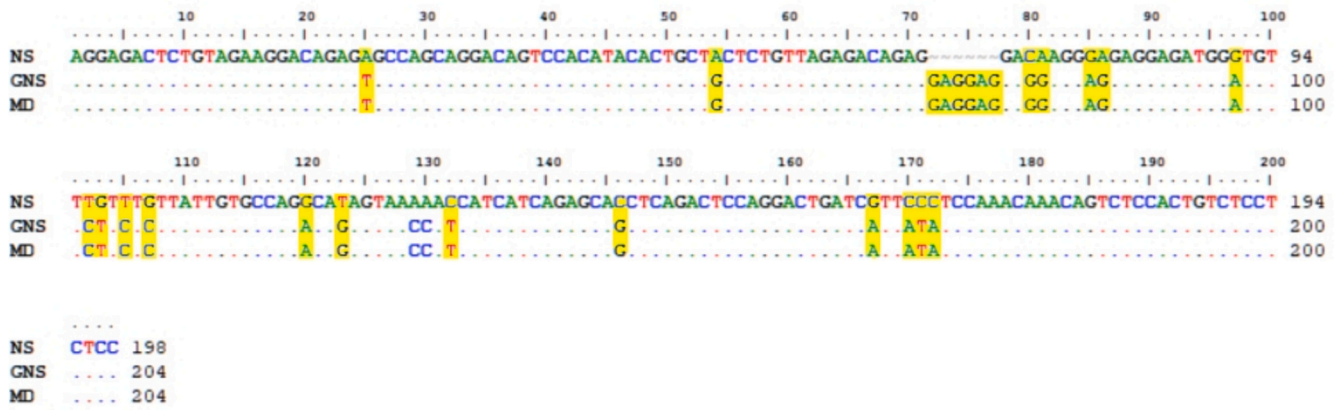


Fig. 6. Nucleotide sequence alignment of the microsatellite DNA for NS, GNS, and MD. (The yellow background represents bases where the GNS and MD bands differ from NS, and the dots represent bases that are identical to NS.) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

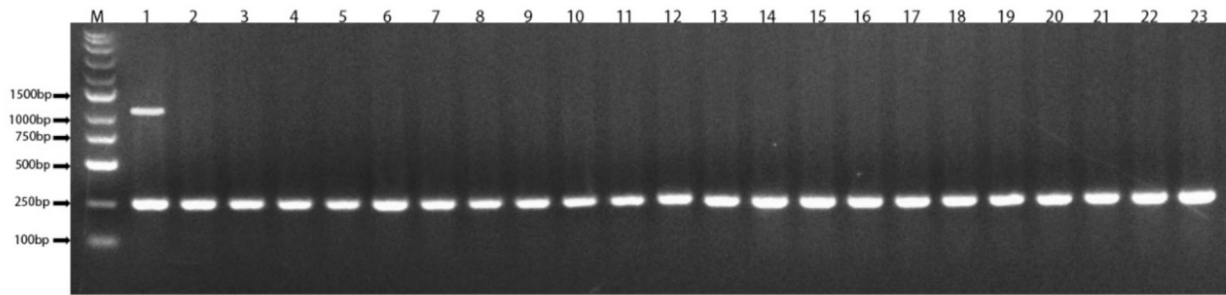


Fig. 7. Sexual identification of GNS by sex markers. Lane 1 represents the male control, and lanes 2–23 represent GNS. Lane M represents the maker.

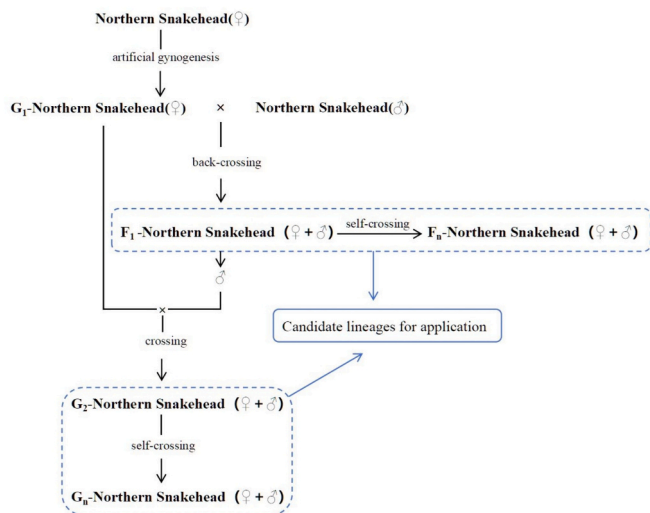


Fig. 8. The process for generating new lineages by artificial gynogenesis in the northern snakehead. Our team has completed the establishment of the F₁-NS population in 2024, and we will expand the population by self-crossing F₁-NS. At the same time, we will generate a G₂-NS population by crossing.

the second polar body (Jin et al., 2022; Morishima et al., 2011). Simultaneously, it is essential to reach the ideal water temperature for activation. Our study found that many researchers ignore the incubation water temperature in the intermediate stage of post-fertilization development time. After continued experimentation, we have found that the temperature of the incubation water at this stage significantly affects the discharge of the second polar body. Lastly, the temperature and duration

of cold shock treatment for inducing artificial gynogenesis in different fish species vary significantly (Morishima et al., 2011; Samonte-Padilla et al., 2011). These are reflected in the Table 1.

The study showed that the morphology of GNS and NS was the same. At the same time, there were significant differences with MD, which indicated that GNS inherited the main morphological characteristics of NS. The DNA contents of GNS and NS were highly consistent, and both were in the diploid state. In addition, chromosome number and karyotype analyses showed significant homogeneity in chromosome number and structure between GNS and NS. The experiment results are consistent with previous fish produced through artificial gynogenesis (Gong et al., 2019; Wu et al., 2023). These results highlight that the progeny produced through the artificial gynogenetic technique mainly inherited the genetic characteristics of the mother (Samonte-Padilla et al., 2011). These findings not only enhance our understanding of the genetic characteristics of GNS but also provide a crucial scientific basis for further germplasm improvement and genetic studies.

Microsatellite profiling revealed that GNS carries a unique band from the MD. The unique band may be a DNA fragment from the MD sperm that was randomly integrated and inserted into the nuclear DNA of the egg. Artificial gynogenesis is a unique micro-hybridization technique that implants specific sperm DNA fragments into the genetic material of the fish. We know from previous studies that distant hybridization can produce progeny with significant heterozygous advantages by combining advantageous traits from different species, sometimes even leading to the formation of new species (Liu, 2010; Wong et al., 2022). Allo-sperm fragments are often present in many fishes produced by gynogenesis, which may explain why these gynogenetic fish have dominant traits that are not present in the maternal generation. Previous studies have shown that hermaphrodite bluntnose seabream induced with MD sperm outperformed conventional bluntnose regarding growth

rate, muscle texture, and antioxidant capacity (Gong et al., 2019). Artificial gynogenesis induced by largemouth bass in mandarin fish induced significantly faster embryonic development than in mandarin fish (Wu et al., 2023). Artificial female nuclear development is a unique micro-hybridization technique that implants specific sperm fragments into the genetic material of the haploid. Grass carp induced by koi carp sperm and their backcross progeny exhibited enhanced disease resistance compared to ordinary grass carp (Mao et al., 2020). In our study, we discovered an improved growth rate in GNS. These results are consistent with previous research and further endorse the importance of gynogenesis in fish breeding. The GNS exhibited a 6.5% increase in body length and a 15.8% increase in body weight compared to the NS in 3 months. In our cultivation of GNS, we have observed that GNS exhibits markedly superior growth compared to NS, a phenomenon potentially attributable to the integration of MD DNA fragments into the GNS genome. However, additional data are required to substantiate this characteristic. Therefore, our research will continue in this direction.

This research, employing sex-specific molecular markers, has validated that NS induced with MD sperm can successfully produce all-female offspring (GNS), aligning with the results exhibited in previous studies (Honji et al., 2022; Matsuda et al., 2002; Zhou et al., 2000). In conclusion, we have pioneered the production of a new type of gynogenetic NS by triggering artificial gynogenesis in NS with UV-inactivated MD sperm. Through detailed investigations of DNA content, chromosome count, karyotyping, and genetic composition of GNS, we ascertained that GNS constitutes a diploid fish species, harboring 48 chromosomes. The genetic constitution of GNS closely resembles that of its maternal lineage, augmented by the integration of DNA fragments from MD. It displays a growth advantage-illustrating the “allo-sperm effect.”

Furthermore, sex-specific markers have confirmed GNS as an exclusively female population. Normal development of gonads and other organs in GNS observed by dissecting GNS after 10 months of aquaculture suggests that heterologous sperm from different families successfully induce the gynogenesis of NS. This study provides a new technique for NS breeding and lays a scientific foundation for understanding the biological mechanism of gynogenesis and improving fish breeding methods. Future research should investigate the intrinsic mechanism of gynogenesis and enhance the breeding strategy. This strategy will help achieve sustainable development and efficient use of fish resources.

CRedit authorship contribution statement

Anmin Liao: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Shuxin Zhang:** Writing – review & editing, Writing – original draft, Data curation. **Qiuyan Yu:** Formal analysis, Data curation. **Yude Wang:** Formal analysis, Data curation. **Huifang Tan:** Formal analysis, Data curation. **Ping Wu:** Writing – original draft, Data curation. **Yue Ding:** Writing – review & editing, Writing – original draft, Data curation. **Biao Hu:** Data curation. **Wuxia Liu:** Data curation. **Min Tao:** Writing – review & editing. **Chun Zhang:** Writing – review & editing, Writing – original draft, Data curation. **Qinbo Qin:** Writing – review & editing. **Kaikun Luo:** Data curation. **Chenchen Tang:** Data curation. **Qizhi Liu:** Writing – review & editing, Writing – original draft, Visualization, Validation, Formal analysis, Data curation, Conceptualization. **Shaojun Liu:** Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare no competing interests.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.aquaculture.2024.741488>.

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