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泥鰍、鯉魚及九孔之染色體操作

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Studies on Chromosome Manipulation in Cyprinid Loach (*Misgurnus anguillicaudatus*), Common Carp (*Cyprinus carpio*), and Small Abalone (*Haliotis diversicolor*)

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INTRODUCTION

With the aim of establishing chromosome technologies in practical work of fish farming, ploidy manipulation has been studied in various finfish and shellfish in Taiwan. Both cyprinid loach (*Misgurnus anguillicaudatus*) and common carp (*Cyprinus carpio*) are very important freshwater fish farming and have been the target species for triploidy manipulation. Inbred lines are traditionally produced by repeated full-sib mating. However, cyprinidae have relatively long generation intervals. For this reason, full-sib mating is not the method of choice. Instead, gynogenesis has been adopted as a quick method to produce all female inbred strains.

Small abalone (*Haliotis diversicolor*) is a unique shellfish with about 10% of its total weight being non-attractive colored gonad during its spawning season (Chao et al., unpublished). Feasibility of ploidy manipulation to reduce the gonad somatic index for a larger edible part and better flavor needs to be verified. Triploid production has been reported to be a favorable method of obtaining sterile strains and hence, a means of increasing adult size and avoiding loss of glycogen in spent gametes.

This paper summarizes optimal conditions to induce gynogenetic diploids and triploids and practical methods to determine the presence of triploid in these species that were developed since 1985 and 1990 respectively, for finfish and shellfish in our laboratory.

MATERIALS AND METHODS

A. Strategies in Retaining Polar Body or Inhibiting Cleavage

To induce triploidy in a variety of aquatic organisms, thermal shock, hydrostatic pressure shock and chemical shock have been applied to suppress polar body formation or to block mitosis. The efficiency of these shocks depends on three main parameters: (1) shock

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conditions (cold or heat temperature in thermal shock, pressure intensity in hydrostatic pressure shock, kind and concentration of chemical solution in chemical shock); (2) the duration of shock; and (3) its timing. Selection and determination of optimal starting time were carried out due to a very rapid chromosomal and cytoplasmic changes during early embryogenesis.

A-1. Cyprinid Loach.

Female spawners were selected from broodstock under captivity and injected with pituitary gland or HCG. Ripened eggs were stripped out from gravid females. The testes were removed from one mature male and ground with Ringer solution for freshwater fish. Sperm was activated and mixed with eggs immediately. Loach eggs were exposed to 8 ℃ for 40 min; the starting time of cold shock treatment varied from 1 to 9 min after fertilization to find the best timing. The eggs were exposed to various temperatures of 1, 4, 6, 8, and 12 ℃ for nine duration times of 5, 10, 20, 30, 40, 50, 60, 70, and 80 min to determine the optimal combination of cold shock conditions (Chao et al., 1986).

Breeding of golden loach was attempted by means of induced gynogenesis. The female broodstock, golden in phenotype, were stripped for eggs after induced maturation. Milt from common loach was genetically inactivated by UV irradiation at a dosage of 1-2J/cm2, or 0.05W/cm^2 for 20-40 seconds. The eggs fertilized with the inactivated sperm were subjected to cold shock of 6℃ for 20-50 min or 8℃ for 40-60min and pressure shock ranging from 500 to 12,000 psi for 2-5 min, beginning at 5 min after fertilization (Chao & Liao, 1990).

A-2. Common Carp.

Male spawners with running milt and female spawners under captivity with soft belly were selected from broodstock during their propagation season, from February to April. They were kept in a small pond maintained with running water sourced from the underground for egg maturation and spawning. Artificial propagation was done. Fertilized eggs were sprayed on net or put in a pressure cylinder. The experimental treatments for inducing triploidy included cold shock of 1 and 3 ℃ and pressure shock of 650 kg/cm^2 at 1, 3, and 5 min after fertilization.

Most recently, gynogenetic diploid induction mainly by either heat shock of 40 ℃ for 2 min starting at 24-30 min to block mitosis or cold shock of 1 ℃ for 30 min starting at 1-5 min to retain second polar body in fancy carp has been under preliminary field trials by using the 100x diluted and UV-irradiated sperm during fertilization and referring the treatment parameters obtained in triploid induction (Hollebecq et al., 1986; Taniguchi et al., 1990; Rothbard, 1991).

A-3. Small Abalone.

Spawners with visible full whitish testis or brown greenish ovary were selected in late October and November. In separate tanks, they were treated wuth water heated from the original water temperature up to 4-6 ℃ (however, never above 30 ℃) at a gradually increasing rate of 1 ℃/h. The heater was then turned off, allowing the temperature to drop normally. During the late phase of 1st and 2nd cycle, some male and some female spent sperm and eggs. Milt was added into the egg tank at an optimum ratio for artificial fertilization.

For comparison, 21-42 min after fertilization, ideally before the 1st or 2nd polar body was extruded, the fertilized eggs were immersed in a solution of 0.5-1mg/l cytochalasin B dissolved in 0.1 % DMSO for 20 min (Allen et al., 1988; Beaumont & Fairbrother, 1991). The eggs were then rinsed with 0.1 % DMSO solution and seawater before being stocked in outdoor ponds equipped with a series of corrugated plastic sheets for spat resting .

B. Assessment of Triploidy Presence

B-1. Microfluorometry.

Blood smear of at least 20 day old fry or larvae were made on slides and fixed in methanol-formalin 9:1 V/V for 15 min. Slides were washed twice with distilled water, dried and stained by the Feulgen reaction procedure (Humason, 1979) or DAPI (Komen et al., 1988).

The DNA content of erythrocyte nuclei was measured (10 nuclei per fry) with Leitz MPV 3 microscope photometer (Gervai et al., 1980; Johnstone, 1985). Each nuclei was read 32 times automatically. The readings of stains vary with DNA content in the erythrocyte nuclei and variation between and within each batch of stained slides. The mean value of readings of each sampled fry in experimental and control group were corrected against the corresponding quantitative blank smear on the same slide to exclude the variation between and within each batch of stained slides. Since the control groups produced only diploids, triploidy was acknowledged when the corrected reading on a slide of sample fry was about 1.5 times that of the mean reading of the control group. The histograms of fluorescence intensity representing relative DNA content were constructed for comparison. Triploid, diploid, and haploid cells show three distinct peaks.

B-2. Flow Cytometry.

Saturated EDTA solution just enough to coat a thin layer on the inner walls of a 1 ml syringe was used to treat red blood cells to avoid coagulation. PBS-BSA-NaN_3 solution was added to bring the mixture up to 1ml. The mixture was kept at 4 ℃ before analysis. Protocols A, B, and C were compared (see Appendix for the schematic diagrams of the

Appendix

RPOTOCOL A

0.2 ml RBC sample in Heparin or saturated EDTA, add PBS (pH 7.4)

↓

Centrifuge 1,500 rpm, 10 min, discard supernatant (wash)

↓

Treat with 0.1% Triton-X-100 0.15 M NaCl (4 ℃), 5 min at Triton-X-100 solution: RBC=1:1

↓

Wash with Pbs, 2x

↓

Add 100 μ l 1 ma/ml Rnase (DNA free) + 100 μ l, 400 μ g/ml Propidum iodide (keep in the dark)

↓

Incubate, 37 ℃, 30 min

↓

Wash with PBS, 2x

PROTOCOL B

Centrifuge RBC sample, 1,100 rpm, 5 min

↓

Discard supernatant

↓

Add solution A 30 μ l, incubate, 37 ℃, 20 min

↓

Add solution B 30 μ l, treat, 4 ℃, 1h

SOLUTION A

0.5 ml Propidium iodide stock solution
0.5 ml Rnase stock solution
0.1 ml Triton-X-100 stock solution

SOLUTION B

0.5 ml Propidium iodide stock solution
0.1 ml Triton-X-100 stock solution
9.4 ml 0.4 M NaCl solution, adjust to pH=7.2

PROTOCOL C

0.2 ml RBC sample on saturated EDTA, add 0.8 ml PBS-BSA-NaN3 solution [(1% BSA + 0.05% NaN3) in PBS]

↓

Resuspend cells, fiter though 40/60 μ m net

↓

Wash with PBS, 2x, spin 1,500 rpm, 30 sec

↓

Set cell density = $8 \times 10^5 - 1 \times 10^6$ cells/ml

↓

Add 20 μ l of 5% Triton to 0.5 ml cells in PBS

↓

Add 20 μ l of Propidium iodide (2.5 mg/ml) protocols).

In protocol B, as an example, the mixture was centrifuged at 1,100 rpm for 5 min. After the supernatant was discarded, the cells were treated with 0.3ml solution A (0.5ml RNase + 0.1ml Triton X-100 + 0.5ml Propidium iodide) and incubated at 37 ℃ for 20 min. It was then treated with 0.3ml solution B (0.5ml Propidium iodide + 0.1ml Triton X-100 + 9.4ml 0.4M NaCl) and incubated at 4 ℃ for one hour. The samples were protected from light until the flowcytometric analysis using FPICS Profile II (Coulter Corporation) was processed. The optimal injection flow rate of 15-30 ul/min, sheath pressure of 7.51-15.00psi, and laser power 0.15 MV and 8.62 A were suggested to obtain better half peak coefficient of variation and histograms of fluorescence. Mean value of DNA content in triploid cells was about 1.5 times that of normol diploids and thus distinguishable.

B-3. Chromosome Preparation

Embryo or small fish was first incubated in colchicine to arrest mitosis at metaphase for 4-8h depending on the size. Embryo, gill or other tissue of fish was then treated with hypotonic solution, either 0.005 N potassium chloride, 0.005 N sodium citrite or distilled water. The resulting material was then fixed with Carnoy solution of methanol and glacial acetic acid at 3:1 V/V. The tissues were finely chopped using a scalpel. The cell suspension was spread on a warm slide to from cell rings. Proper staining was accomplished by using Giemsa solution.

Observation through microscope at 600x or higher was followed by photograph taking and counting the number of metaphase chromosome. If necessary, the chromosome may be cut individually and the cutouts pairing homologues arranged by size, shape, and length of arms. Each fish with chromosome numbers ranging from 1.2 to 1.5 times that of normal diploid fish was considered to be triploid. The percentage of triploid fish was, therefore, counted.

RESULTS

Chromosome manipulations have been carried out in three local aquatic species in Taiwan-loach, carp, and small abalone -using thermal, pressure and chemical shocks. Table 1 sets out the methods which have been used for induction and ploidy assessment, together with the best results obtained. The proportion of diploidy and triploid types may change during development when mortality is not avoidable.

When eggs of cyprinid loach and common carp were subjected to cold shock (1 ℃ for 30-40 min in loach, and 1 ℃ for 30 min in carp), those shocked, respectively, at 5 min and 1-3 min after fertilization produced the highest proportion of triploid fry. In cyprinid loach,

Table 1. Chromosome manipulation in cyprinid loach (*Misgurnus anguillicaudatus*), common carp (*Cyprinus carpio*), and small abalone (*Haliotis diversicolor*)

Species	Induction Method	Verification Method	Best % of		Suggested Timing and Duration
			G2N	3N	
Cyprinid Loach	CS	mf		80-100	5 m.a.f., 1 ℃, 30-40 min
	CS	cm	70		3 m.a.f., 6 ℃, 30 min
	PS	cm	32		5 m.a.f., 6000 psi, 4 min
Common Carp	CS	fc		66-86	1-3 m.a.f., 1 ℃, 30 min
	PS	fc		7.7	5 m.a.f., 650 kg/cm^2, 10 min
	HS	--	--		1-3 m.a.f., 40 ℃, 2 min
Small Abalone	CS	cp		0	
	CB	cp		22-50	24-39 m.a.f., 20 min

3N =Triploid. G2N=gynogenetic diploid. CB=cytochalasin B. CS=cold shock. HS=heat shock. PS=pressure shock. cm=color marker.
cp=chromosome preparation. fc=flow cytometry. mf=microflurometry. m.a.f.=minute after fertilization.

triploidies of 80.8, 80.0, 95.0, 100.0, and 100.0 were obtained in the groups treated at 1 ℃ for 60, 20, 50, 40, and 30 min starting at 5 min after fertilization. In common carp, triploidies of 66.6, 80.4, 86.2, and 86.6 % were obtained in the groups treated at 1 ℃ for 30 min starting at 1-3 min after fertilization.

When eggs of cyprinid loach, golden loach, and common carp were first fertilized with irradiated sperm and then subjected to cold shock, morphologically normal fry were confirmed to be diploid. In cyprinid loach, optimum cold shock of 6-8 ℃ for 30-40 min at 3-5 min after fertilization resulted in comparatively high percentages of normal fry. However, the survival rate was low in the diploid gynogenetic group of cyprinid loach. In golden loach, the percentage of golden fry reached 70 % but the survival rate was also quite low. In fancy carp, preliminary results showed that heat shock of 40 ℃ for 2 min to inhibit the first mitotic cleavage gave much higher hatching in most cases than cold shock of 1 ℃ for 30 min to retain the second polar body. The induction of a desirable color pattern was far from being successful despite the adoption of properly irradiated sperms of common carp, loach , or catfish. The experiment is still undergoing to deal with this. Problems such as the high cost of pure strain spawners and the difficulty in categorizing the fingerling of various color patterns need to be solved with the advice of experienced scientists. Various reasons in failure of inducting the desirable color pattern of fancy carp using gynogenensis induction will be determined and minimized.

In small abalone, treatment with cytochalasin B at 1mg/l for 20 min started at 24, 27, 30, and 39 min after fertilization gave a chromosome counting of triploid of 33.3, 22.2, 50.0 and 35.7%. However, no significant results were obtained with thermal shock trials.

The three methods used in triploidy identification have their own particular characteristics (Table 2). In instruments used, microfluorometry (MF) makes use of the fluorescent microscope; flow cytometry (FC), the flow cytometer and chromosome preparation (CP), the light microscope. In the target material to be studied, MF and FC aim for the DNA content in the red blood cell, while CP, the chromosome number in the cellar nuclei of the embryo, gill or cell line. In the possible errors when using the different methods, MF has focusing and condenser errors; FC, poor pre-treatment and inconsistent laser power; and CP, overlapping of and missing chromosome during preparation. In the number of tested cells per individual finfish and shellfish , MF has 30-10 cells; FC, >10,000 cells; and CP, 30-100 cells. In pre-treatment time per individual, MF requires 40 min; FC, 3 min; and CP, 3-12 h. Lastly, in either observation or sample run time per individual, it takes about 1 h for MF; 2 min for FC; and 1 h for CP. Protocols A, B, and C were equally functional.

The choice of method in triploidy identification depends on the number of fish, effort involved, availability or cost of instruments and the desired level of accuracy.

Table 2. Comparison of three methods of polyploid determination

	Microfluorometry	Flow Cytometry	Chromosome Preparation
Tool Used	fluoresence microscope	Flow Cytometer	Common microscope
Sample Taken	RBC	RBC, Tissue	Embryo, Cultured cell, Tissue
Target Material	DNA content	DNA content	Chromosome number
Possible Error	Staining difficulties	Poor treatment, Inconsistent laser power /mercury lamp intensity	Overlapping of and missing chromosomes
Sampling Number	30 ± 10 cells/slide	>100,000 cells/vial	30-100 cells/slide
Time for Pretreatment/individual	40 min	3 min	3-12 h
Time for Observation/individual	1 h	2 min	1 h

DISCUSSION

Triploids can not synapse at meiosis and, therefore, are expected to be sterile. Consequently, there are several advantages. Energy and nutrient for gamete production become available for somatic growth in sterile triploid. The flavor of tripolid may be improved due to storage of glycogen. Sterile triploids of introduced species may be cultured or stocked in water which might be sensitive to the accidental introduction of competitor spices. On the other hand, gynogenesis is believed to be an efficient method for the rapid production of highly homozygous inbred lines. Chromosome manipulation in finfish and shellfish worth further study and progress although the progress of developing the repeatable and reliable techniques takes much time.

Cold shock was found powerful in inducing triploid of finfish while cytochalasin B has proved to be a reliable chemical agent for inducing chromosome doubling during the maturation division in shellfish. Heat shock to block mitotic division in inducing gynogenesis, with a better chance of obtaining high hatching rate and survival rate, in carp was note. Both Hollebecq *et al.* (1986) and Rothbard (1991) also successfully applied heat shock to induce diploid gynogenesis in carp. Further studies should be taken to determine why there are such differences in favorable methods for finfish and shellfish; for meiosis and mitosis manipulation. However, in fancy carp, how hereditary characters, such as color patterns and spot location are inherited from generation to generation has not been well studied. Inbreeding of fancy carp from the population we obtained the spawners for induction test of gynogenetic diploid using mature male and female of same color pattern gave non-predictable color pattern at a random composition in their offspring. Before chromosome manipulation techniques can be applied to produce a good number of fancy carp with desired color pattern, there is much to be studied.

All the currently recommended artificial treatment caused some percentages of abnormalities and increased mortality during early development. Scientific approach to deal with the concerned mechanism and the possible alternative method with less damage is doubtlessly needed.

Commerical triploidy or gynogenetic diploidy is not yet available for either one of these three experimental species. Development of chromosome manipulation in both finfish and shellfish should continue to expand in the future to make marketing possible to explore their valuable feature.

After using both microfluorometry and chromosome counting for assessing ploidy, the disadvantages of either being rather intricate and time consuming or having much routine and tedious staining and microscopy observation job were noted. They may be useful to determine ploidy in a limited number of individual. Nevertheless, when assessment of ploidy percentage of several populations of finfish and shellfish are undertaken within several days using traditional methods, the majority of cells examined on a slide did not often provide really countable chromosome, particularly in the fish with chromosomes more than 50.

The identification of ploidy using various pre-treatment protocols to stain DNA of suspensible cells with specific fluorescent dye and flow cytometer to determine DNA content has the advantages of being accurate to read directly the target material - DNA content of each single cell from same individual. The capability of checking more than 10,000 cells within 3 min and providing the printed data to give convincing evidence make it an effective and recommendable instrument to meet the modern biotechnology era. Besides, a much less expensive flow cytometer (e.g., PARTEC CA II) an alternative instrument equipped with mercury lamp instead of a laser one and with limited specific functions including DNA content measurement than the multifunctional one used for medical research is recently commercially available and is adequate for the purpose of verification of ploidy.

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REFERENCES

1. ALLEN, S.K., Jr., DOWNING, S.L., & CHEN. K.K. 1988. Hatchery Manual for Producing Triploid Oysters. Washington Sea Grant Publication, University of Washington, Seattle, Washington, U.S.A. 27pp.
2. BEAUMONT, A.R. & FAIRBROTHER, J.E. 1991 Ploidy Manipulation in Molluscan Shellfish: A Review. J. of Shellfish Research. 10(1):1-18.
3. CHAO, N.H., CHEN, S.J. & LIAO, I.C. 1986. Triploidy Induced by Cold Shock in Cyprinid Loach, *Misgurnus anguillicaudatus*. IN: J.L. Maclean, L.B. Dizon, L.V. Hosillos (Eds.) The First Asian Fisheries Forum. Asian Fisheries Society, Manila, Philippines. pp. 101-104.
4. CHAO, N.H., LIANG, W.H., Tsai, H.P., & LIAO, I.C. Triploidy Induced by Cytochalasin B Shock in Small Abalone, *Haliotis diversicolor*. (unpublished).
5. CHAO, N.H., & LIAO, I.C. 1990. Production of Golden Loach, *Misgurnus anguillicaudatus* by Means of Gynogenesis. IN: R. Hirano & I. Hanyu (Eds.). The Second Asian Fisheries Forum. Asian Fisheries Society, Manila, Philippines. pp. 535-538.
6. GERVAI, J., MARIAN, T., KRASZNAI, Z., NAGY, A. & CSAYNYI, V. 1980.

Occurrence of Aneuploidy in Radiation Gynogenesis of Carp, *Cyprinus carpio. L.* J. Fish Biol. 16:435-439.

7. HOLLEBECQ, M.G., CHOURROUT, D., WOHLFARTH, G., & BILLARD, R. 1986. Diploid Gynogenesis Induced by Heat Shocks After Activation with UV-irradiated Sperm in Common Carp. Aquaculture. 5(4):69-76.
8. HUMASON, G.L. 1979. Animal Tissue Technique. 4th Edition. W.H. Freeman and Co., San Francisco. pp. 661.
9. JOHNSTONE, R. 1985. Induction of Triploidy in Atlantic Salmon by Heat Shock. Aquaculture. 49:133-139.
10. KOMEN, A., UCHIMURA, Y., IEYAMA, H., & WADA, K.T. 1988. Detection of Induced Triploid Scallop, *Chlamys nobilis*, by DNA Microfluorometry with DAPI Staining. Aquaculture. 69:201-209.
11. ROTHBARD, S. 1991. Induction of Endomitotic Gynogenesis in the Nishiki-Goi, Japanese Ornamental Carp. Bamidgeh. 43(4):145-155.
12. TANIGUCHI, N., YAMASAKI, I., & SATO, M. 1990. Production of the Gynogenesis Diploids Produced by the Cleavage Inhibition Type in Nishikigoi and the Necessity of the Demonstration. Abstract of the Spring Conference of the Japan Fisheries Association. pp.54.

泥鰍、鯉魚及九孔之染色體操作

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摘要

利用染色體操作方式在產生配偶體時使之形成不孕的三倍體個體，可使魚貝類精巢或卵巢萎縮而避免肝醣的損失以達到增加可食用部份及提高風味之可行性。

另外本文報告本實驗室 1985-1990 年間最適誘發雌核生殖二倍體或三倍體之條件和實際的步驟以及探討如何鑑定三倍體之存在，目的在測知泥鰍、鯉及九孔三種供試水產生物其三倍體之誘發成功率。

迄今已建立三種方法，對三倍體實際鑑定而言各有優缺點。在儀器的使用方面，microflurometry (MF)，是利用螢光顯微鏡。Flow cytometry (FC)是利用流式細胞儀。Chromosome preparation (CP)是利用光學顯微鏡。就目標物言，製備染色體時，MF 與 FC，是針對血球細胞中 DNA 之含量 CP 則為胚體細胞、鰓細胞或細胞株中核內染色體套數。至於誤差方面，MF 有對焦與聚光等之可能誤差；FC 其前處理麻煩及雷射效率不穩定等可能產生誤差；CP 製備時會有染色體重疊和丟失的可能誤差。對不同種別單一個魚、貝類可一次測試細胞之數量來看，MF 可有 30 ± 10 cells；FC，>10000 cells；CP, 30-100 cells。在各別之前處理時間上，MF 需 40 min，FC 需 3 min，CP 則需 3-12 hr。最後對觀察或樣品進行測試的時間而言，MF 得花 1 hr，FC 只需 2 min，CP 亦約為 1 hr。本文所列三種流程 A、B、C，具有相同的功能。

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