MORPHOLOGY, CHROMOSOME COUNTS AND GENOMIC ANALYSIS OF ABNORMAL HAPLOID OFFSPRING OF CLARIIDS (*CLARIAS MACROCEPHALUS*)

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ABSTRACT :- Morphological abnormality (haploid syndrome) of fry was obtained from crosses between ultraviolet-irradiated (0.3 J/ cm^2) eggs of *Clarias gariepinus* and fresh sperm of *C. macrocephalus*. The abnormal fry had a short, curved tail and an enlarged cavity between the yolk sac and anterior part of the body. Chromosome counts and analysis of genome size indicated that the abnormal fry were haploid, and amplification by polymerase chain reaction of genomic DNA using primers targeting a portion of the gene encoding the immunoglobulin heavy chain confirmed inheritance of the paternal genome. These findings will aid in distinguishing diploid from haploid androgenetic offspring.

Key words : karyotype, genomic analysis, haploid syndrome, *Clarias macrocephalus*

INTRODUCTION

Induced haploid androgenetic offspring can be produced from genetically inactivated eggs fertilized with normal sperm (Ihssen et al. 1990). Generally, these offspring do not survive to hatching, but recent attempts to induce diploidy by suppressing the first mitotic cleavage have been successful in *Cyprinus carpio* L. (Bongers et al. 1994). The eggs were UV-irradiated and fertilized with normal sperm and shocked to restore diploidy.

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Experimental radiation dosages are usually optimized by trial and error, and success of genetic inactivation of the eggs is estimated by percent production of abnormal (putative haploid) embryos. Complete genomic inactivation of the eggs would result in 100% haploid embryos. However, while attempting to standardize the dosage of UV irradiation of *Clarias gariepinus* eggs in our laboratory, normal and semi-abnormal embryos were observed probably due to incomplete inactivation. Therefore, reliable methods for ploidy identification are required for standardization of irradiation of fish gametes. Many methods can be used, such as chromosome counts, and nucleolar counts (Phillips et al. 1986). Although the latter method can only be applied to species that have one chromosome with a nucleolar organizer region per haploid genome (Ihssen et al. 1990). Flow cytometry is also an accepted method of identifying ploidy of fish by measurement of the DNA content of individual cells (Allen 1983; Tiersch and Chandler 1989).

In an attempt to save endangered species of Thai fish, such as the Mekong giant catfish, *Pangasius gigas*, different androgenetic techniques have been evaluated. Eggs of *C gariepinus* were exposed to ultraviolet radiation and fertilized with sperm of *C. gariepinus, C. macrocephalus*, or of *P. gigas*. Because *P. gigas* sperm are difficult to obtain, crosses of UV-irradiated *C. gariepinus* eggs with sperm of *C. macrocephalus* or *P. hypophthalmus* were performed for standardization of UV exposure.

Presently, we have been able to identify genomic differences in catfish of the family Clariidae (*C. macrocephalus, C. gariepinus* and their hybrids) and *Pangasiidae* (*P. gigas, P. hypophthalmus* and the hybrids) by polymerase chain reaction using DNA primers (Thongpan et al. 1997) designed to target the CH4 exon of the channel catfish (*Ictalurus punctatus*) gene encoding the constant region of the immunoglobulin M heavy chain (Zhang et al. 1994).

In this study, we demonstrated that morphological observation was a simple and reliable method to distinguish haploid from diploid or polyploid fry, by comparing the morphological method with: 1) chromosome counts; 2) measurement of genome size; and 3) polymorphic DNA patterns of abnormal (putative haploid) and normal (putative diploid) fry obtained from crosses between UV-irradiated (0.3 J/cm^2) eggs of *C. gariepinus* and fresh sperm of *C. macrocephalus*.

MATERIALS AND METHODS

Egg and sperm samples

Catfish used in this study were *C. macrocephalus* and *C. gariepinus*. They were of domesticated pond-raised stock from the Department of Aquaculture, Faculty of Fisheries, Kasetsart University, Bangkok. In each trial, three females (*C.*

gariepinus) were induced to spawn by intra-muscular injection of 30 µg/kg of luteinizing hormone-releasing hormone analogue (LHRHa) (Suprefact, Holscht AG, Main, Germany) and 10 mg/kg of domperidone (Motilium, Olic, Bangkok, Thailand). Egg samples were collected by abdominal stripping 12 h after injection. The sperm samples from 2 to 3 males were obtained by removing the testis and crushing them in plastic bags containing sodium chloride solution (0.9%, 300 mOsm/kg) to yield a 1:1 dilution ratio (w/v). The sperm cells were separated from tissue debris by careful pipetting. In all cases, only samples with ≥ 50% motile sperm were used.

UV-irradiation of eggs

Egg samples (~100 eggs/determination) of *C. gariepinus* were pipetted and spread in round-bottom plastic cups (20 x 35 mm) to form a monolayer before UV exposure. The exposure (0.3 J/cm^2) was done for 6 sec at 35 mm below the light source (40W, Sylvania, Japan).

Fertilization tests

Each aliquot of irradiated or non-irradiated (control) eggs of *C. gariepinus* was fertilized by adding 50 µl of diluted testicular sperm (~7x10^6 cell/egg) from *C. macrocephalus* (interspecific hybridization). After the eggs and sperm were mixed, 600 µl water was added to activate the gametes and they were incubated at 26-27 °C in 1.5 l of dechlorinated tap water with continuous aeration. Fertilization success was assessed as the percentage of embryos with eyecup development at 20-24 h after insemination. The percentage of hatching and abnormal fry were observed 24 h later (~48 h after fertilization). Samples of abnormal (n = 10) and normal (n = 10) fry were collected and photographed after thorough examination with a stereomicroscope (at 10x).

Chromosome spreading

Preparation of chromosomes for counting was done according to the technique described by Baksi and Means (1988). Live embryos or fry were exposed to colchicine (0.05%) for 4 h in 2-ml cryotubes. The organisms were removed from the exposure tube and placed into a tube of hypotonic solution (0.5% KCl) for 90 min. The hypotonic solution was decanted and replaced with a freshly prepared 3:1 mixture of methanol:glacial acetic acid. The first exposure with this fixative was for 30 min, followed by an overnight exposure. The samples were washed with fresh fixative for 30 min before transferring to glass slides. The tissues were macerated using fine-tip forceps in 60% acetic acid. The cell suspension (5 drops/individual) was dropped from the height of 75 mm onto ethanol-cleaned 45°C slides. The slides were allowed to dry for 1-2 d before staining with 10% Giemsa solution for 20 min. Metaphase chromosomes were examined and photographed at 1,000X.

Polymorphic DNA patterns

Samples of fry were placed in cryotubes and plunged into liquid nitrogen for storage until further analysis. All glassware, pipette tips and solutions were autoclaved to avoid DNAase contamination. Molecular biology grade reagents were used. DNA isolation was done by placing samples into 2.5 ml microcentrifuge tubes containing 200 µl of DNAZOL reagent (Gibco, BRL, Gaithersburg, Maryland) and were vortexed for 2 min to lyse cells. Five hundred microliters of 95% ethanol was added to the samples, gently mixed for 5 min, and centrifuged at 7,000 g for 10 min to precipitate the genomic DNA. This step was repeated twice to remove debris. The DNA was transferred to 1.5-ml microcentrifuge tubes and air-dried for 3-5 min. Two-hundred microliters of sterilized water was added, mixed well and heated at 45°C in a water bath for 10 h to solubilize the DNA. Samples were centrifuged at 7,000 g for 10 min and the supernatant was collected. Estimated purity (1.8-2.9) and concentration (0.3-0.5 µg/µl) of DNA were measured with a spectrophotometer (Beckman DU series 60) at 260 and 280 nm. PCR primers (CH4) developed for studying channel catfish DNA (Zhang et al. 1994) were used. The primers were designed to target the CH4 exon of the gene encoding the constant region of the channel catfish immunoglobulin M heavy chain, based on the published sequence of the gene (Wilson et al. 1990). These primers were synthesized by the LSU Gene Probes and Expression Systems Laboratory in Baton Rouge, Louisiana. The primer sequences were (5' to 3'): TCCCCAAGGTTTACTTGCTCGCTCC and CGATGGATCTGGATATGTGGCGCAC. From these primers a DNA fragment of about 303 base pairs was expected to be amplified from catfish DNA. PCR was performed in reaction mixtures containing 1 µg of target DNA, 0.5 µl (15-19 µM) of each primer, 5 µl of 10-x PCR buffer, 1 µl of deoxyribonucleotide triphosphates mix (10.0 mM each of dATP, dTTP, dCTP, and dGTP), and sufficient sterilized water to make a final reaction volume of 50 µl. Each reaction mixture was covered with 50 µl mineral oil. The PCR tubes were placed into the thermal cycler (Coy Temp Cycle II, Papst-Motoren, Germany) and denaturated at 95°C for 2 min. Then 1 unit of *Taq* DNA polymerase (Gibco) was added under the layer of mineral oil, and 30 cycles of PCR were performed using the conditions of: denaturation at 95°C for 30 sec; annealing at 59°C for 30 sec; elongation at 72°C for 30 sec. The PCR products were stored at 4°C until analysis by electrophoresis. Each sample (12 µl) was loaded in a 2% agarose gel (Amresco, Solon, Ohio) in TBE buffer (0.089 M Tris, 0.089 M Borate and 0.002 M EDTA) and electrophoresed at 5V/cm for 1.5 h. DNA was stained with 0.5 µg/ml ethidium bromide, and the banding of DNA fragments was visualized and photographed on an ultraviolet transilluminator (Photodyne UV 26, Hartland, Wisconsin). The fragment sizes (number of base pairs, bp) were estimated graphically by the use of a standard curve derived from data on the mobility of a 123 bp ladder DNA marker (Gibco).

Genome size

Fry were collected twice (at 28 h and 3 d), and based on their appearances were placed into three categories: normal, abnormal or semi-abnormal (as described below). The fry were stored in 50% methanol for 6 weeks prior to analysis. Preliminary experiments were performed to increase the yield of analyzable nuclei and reduce debris (data not shown). For isolation of nuclei, individual fry were frozen in liquid nitrogen, thawed at room temperature, and minced with a clean scalpel in a lysis solution containing 0.1% sodium citrate and 0.1% Triton X-100. The sample was pipetted up and down 5 times and centrifuged at 8,000 rpm for 10 min. Supernatant (25 µl) was mixed with blood cells from a channel catfish, *Ictalurus punctatus*, (used as an internal reference) in 0.5 ml of a 50 mg/l solution of propidium iodide containing 25 µl RNAase (1.0 mg/ml), 0.1% sodium citrate, and 0.1% Triton X-100. Nuclear fluorescence was analysed with a Becton-Dickinson FACSCalibur flow cytometer according to the method described in Tiersch et al. (1989). The minimum of 10,000 cells was scored for each sample.

RESULTS

Representatives of typical abnormal hybrid fry, with a short, curved tail and enlarged cavity between the yolk sac and anterior part of the body (cardiac cavity), and normal fry are shown in Figure 1. Semi-abnormal fry had a long, curved tail and slightly enlarged cardiac cavity. In all trials, the eggs of *C. gariepinus* exposed to UV radiation (0.24 to 0.3 J/cm^2) before fertilization resulted in ~10% fertilization and about 6% hatching of 100% abnormal individuals. At lower doses of UV radiation ($\leq$ 0.21 J/cm^2) all three types of fry (normal, semi-abnormal and abnormal) were observed (data not shown).

The chromosome counts of these abnormal fry indicated that they were haploid, with 27 chromosomes (1n) (Figure 2 B). Nuclear DNA content (Table 1) of somatic cells was estimated by flow cytometry. The normal and semi-abnormal fry possessed a diploid DNA quantity (about 2.6 pg DNA/cell) while the abnormal fry possessed about one half of that amount (about 1.3 pg). The amplification of nuclear DNA using primers for the CH4 exon of the gene encoding the immunoglobulin heavy chain yielded a single band of about 300 bp for the abnormal fry (Figure 2, Lanes 1 and 2). Simultaneously, the same primers yielded two DNA bands of about 300 and 340 bp in normal fry (Figure 2, Lanes 3 and 4) similar to those of *C. gariepinus* or the hybrids (*C. macrocephalus* x *C. gariepinus*) as shown in Lanes 5 and 6.

Table 1. *Clarias gariepinus* larvae genome-size.

	Normal		Semi-abnormal		Abnormal	
	28 h	3 d	28 h	3 d	28 h	3 d
n	9	10	11	9	7	4
X	2.7	2.52	2.63	2.76	1.42	1.27
SD (n-1)	0.12	0.12	0.18	0.24	0.10	0.04
minimum	2.42	2.38	2.42	2.49	1.32	1.24
maximum	2.85	2.69	3.01	3.30	1.56	1.33
plody	DIPLOID	DIPLOID	DIPLOID	DIPLOID	HAPLOID	HAPLOID

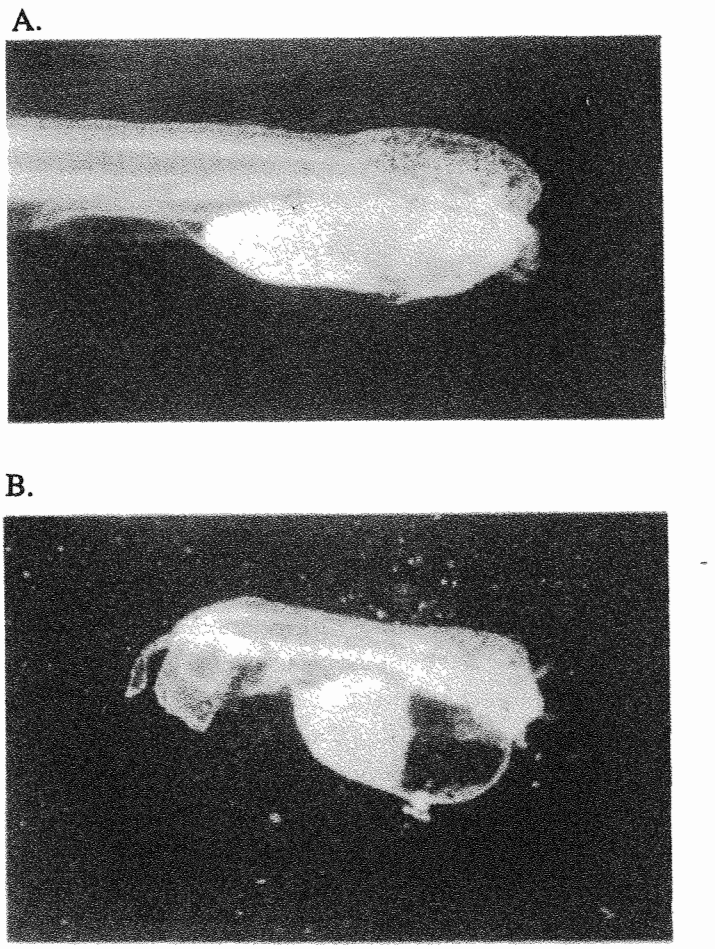

Figure 1. Normal hybrid fry (A) (*Clarias gariepinus* egg x *C. macrocephalus* sperm) and abnormal hybrid fry (B) with haploid syndrome resulting from ultra-violet irradiation (0.3 J/cm^2) of *C. gariepinus* eggs before fertilization with sperm of *C. marcrocephalus.*

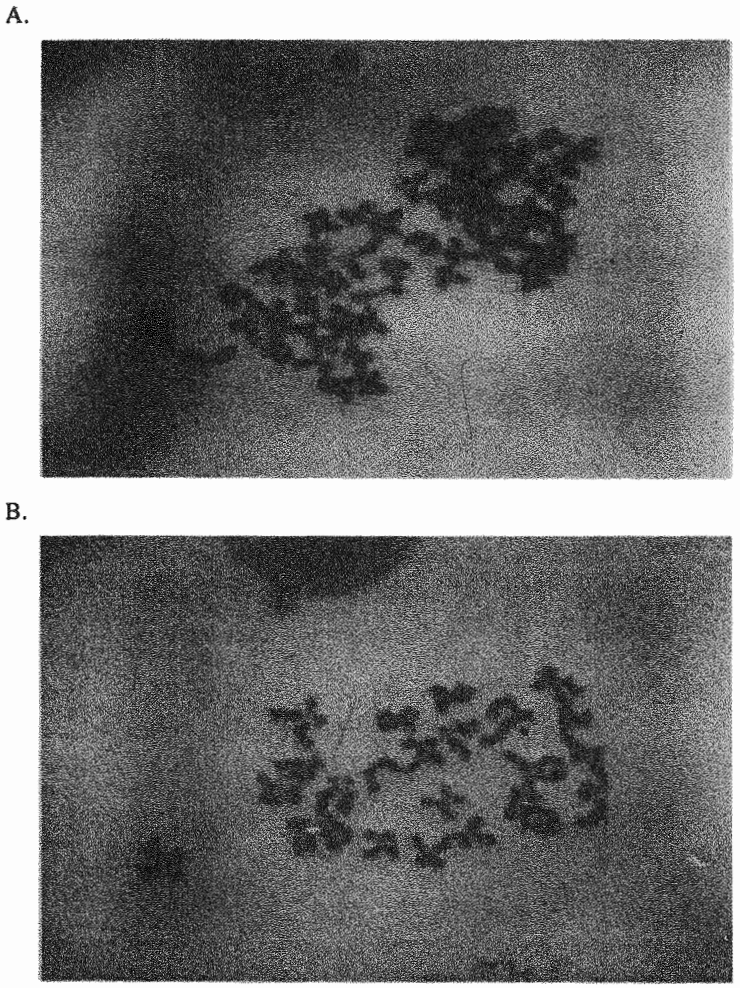

Figure 2. Representative chromosome spreads of the normal hybrid fry (A) with 55 chromosomes (2n) and abnormal fry (B) (see Figure 1) with 27 (1n) chromosomes.

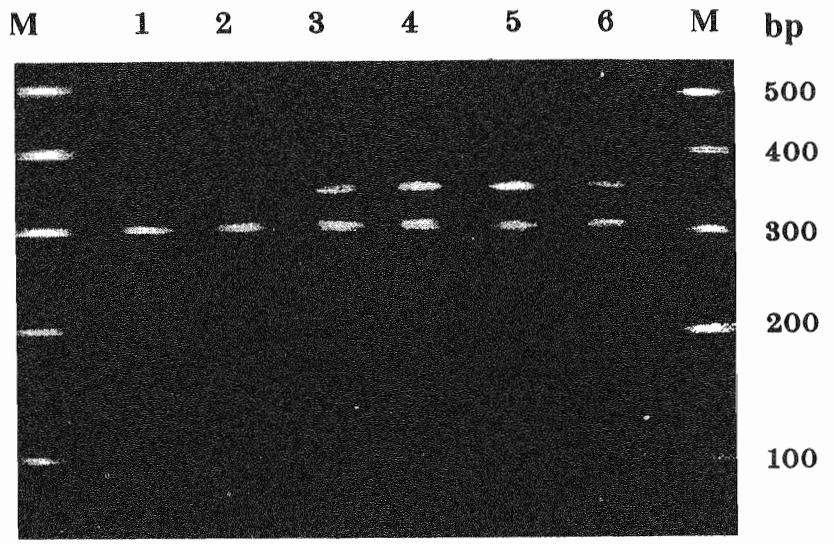

Figure 3. Amplification of genomic DNA from tissues of normal and abnormal fry by polymerase chain reaction. Primer sequences targeted a 300 bp fragment of the CH4 exon of the gene encoding the immunoglobulin M heavy chain of catfish. Lanes 1 and 2, the abnormal hybrids yielded only *C. macrocephalus* DNA banding; Lanes 3 and 4, the normal fry showed two DNA bands (300 and 340 bp) similar to those of *C. gariepinus* or the hybrids, *C. macrocephalus* x *C. gariepinus*, as shown in Lanes 5 and 6 (interassay standard).

DISCUSSION

The viability of haploid fish embryos at various stages of development seems to be species specific. The haploid embryos of medaka, *Orysias latipes*, were unable to develop to hatching (Ijiri and Egami 1980). However, our results show that haploid catfish embryos (*C. macrocephalus*) could develop to hatching but died within 3 d, at which time the yolk nutrients were probably exhausted. In androgenetic or gynogenetic experiments where eggs or sperm were irradiated before fertilization, the viability of fish embryos and fertilization rates respond to radiation dosages according to the "Hertwig Effect". This effect was also observed in UV-irradiation of catfish (*C. gariepinus*) eggs (ranging from 0.03 to 0.33 J/cm^2) before fertilization with sperm of *C. macrocephalus* or *C. gariepinus*. We have found that the theoretically ideal UV dose (0.24 to 0.30 J/cm^2) resulted in about 10% fertilization (data not shown) and 100% abnormal fry showing the haploid syndrome (i.e. short, curved tail with

enlarged cardiac cavity). However, a small number of normal and semi-abnormal fry were also observed in some trials, especially when lower UV doses were applied. Therefore, it was necessary to examine the ploidy of these abnormal and normal fry to ensure their genetic identities at cellular (genome size and chromosome counts) and molecular levels (DNA polymorphism).

From our experience, it was straight-forward to distinguish abnormal fry from normal ones by appearance, even at 1-2 mm length of body size. In additon, we found that abnormal fry swam slowly in circles, while normal fry swam vigorously.

Fish chromosomes are smaller in size than those of mammals. Moreover, chromosome numbers of fish species are large, for example in *Ompok bimaculatus* (2n=92) and *Catlocarpio siamensis* (2n=98) (Na-Nakorn 1995). In clariids, chromosome numbers (2n) vary from 54 (*C. macrocephalus*) to 100 (*C. batrachus*) (Donsakul and Marktoon 1989). Also conflicting reports in chromosome number within the same species are quite common. In *C. batrachus* for example, 104 chromosomes (2n) was also reported by Cha-tung (1991). This variability could be due to natural genetic variation or differences in cytogenetic techniques. In addition, chromosome counts are a laborious method to distinguish haploid, diploid and polyploid fish. This is also true for DNA polymorphic determination. The genomic identification of abnormal fry in this study showed a single DNA band of about 300 bp which corresponded to the 300 bp of CH4 exon of the gene encoding the immunoglobulin heavy chain of *C. macrocephalus* as previously reported by Thongpan et al. (1997, in press). This indicated complete inactivation of the egg genome (*C. gariepinus*) resulting in abnormal haploid fry which inherited only a paternal genome (*C. macrocephalus*). Incomplete inactivation, however, would have resulted in normal fry with two bands (300 and 340 bp) of DNA.

To determine genome size, samples were obtained from entire fry containing a mixture of cell types, thus requiring harsher processing of preparation than that required for fresh blood. These factors can increase the variation observed in genome size values (Tiersch and Wachtel 1993). Even so, we clearly demonstrated that cells of the abnormal fry possessed one half of the DNA content of cells from fry classified as normal or semi-abnormal. From this we concluded that the abnormal fry were haploid, which was consistent with the diagnosis of haploid syndrome associated with the morphological abnormalities observed. Other fry classified as semi-abnormal were diploid but had morphological deformities distinct from those of haploid syndrome. These deformities could have arisen from treatments such as the thermal shock, UV irradiation or genetic incompatibility between the sperm and egg. These results were consistent for fry sampled at 28 h or at 3 d, indicating that androgenetic haploid fry would be identifiable by morphological observation at these times.

The most appropriate and convenient method, therefore, would be to separate fry by phenotypic appearance. Although this is not a fool-proof method, it is less time-consuming and more useful for preliminary screening of individuals, (for example identification of diploid androgenetic offspring). More accurate methods could be applied later to verify the genomic identity of individuals selected for further utilization as broodstock.

The results from this study have shown direct correlations of phenotype, karyotype and genome, of haploid catfish (*C. macrocephalus*). These results could be of value for research in ploidy manipulation in fish, such as gynogenesis and androgenesis.

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