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ISSN 0792 - 156X

PUBLISHER:
Israeli Journal of Aquaculture - BAMIGDEH -
Kibbutz Ein Hamifratz, Mobile Post 25210,
ISRAEL
Phone: + 972 52 3965809
http://siamb.org.il

PRODUCTION OF HETEROZYGOUS AND HOMOZYGOUS CLONES OF COMMON CARP *(CYPRINUS CARPIO* L.): EVIDENCE FROM DNA FINGERPRINTING AND MIXED LEUKOCYTE REACTION

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(Received 10.6.01, Accepted 23.7.01)

Key words: common carp, *Cyprinus carpio,* DNA fingerprinting, heterozygosity, homozygosity, mixed leukocyte reaction

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Abstract

The production of two heterozygous and one homozygous clones of the Israeli Dor-70 line of common carp (*Cyprinus carpio* L.) was tested using DNA fingerprinting (DFP) and mixed leukocyte reaction (MLR) analysis. The clones were obtained in large-scale production from mitotic gynogenetic females and males (sex-inversed). MLR analysis was used to examine major histocompatibility complex (MHC) antigens in individuals. DFP provided evidence concerning identity in the whole genome. Both methods showed that individuals within a progeny were identical, confirming genetic uniformity within each clone and showing that no paternal DNA was transmitted during mitotic and meiotic gynogenesis. The results indicated that uniformity of DFP in progeny of the homozygous clone is obtained only when the mother is fully homozygous, and in the heterozygous clone only if both parents are fully homozygous. The results of both methods provide evidence, though indirect, that the mitotic gynogenetic parents of these fish (mother of the homozygous clone, mother and father of the heterozygous clones) were fully homozygous.

Introduction

Heterozygous and homozygous clones play an important role in developing breeding strategies that aim at fixing novel and superior genes which are desirable for selective breeding and genetic improvement (Streisinger et al., 1981; Nagy et al., 1984; Carter et al., 1991; Quillet et al., 1991; Bongers et al., 1998; Hussain et al., 1998; Galbusera et al., 2000). They are also useful for studies on immunology, disease resistance, sex determination and quantitative genetics. The genetic uniformity of such progeny - putative laboratory clones and/or purelines (homozygous clones) - can be confirmed using methods such as DNA fingerprinting (DFP; Wright, 1993) and mixed leukocyte reaction (MLR) analysis.

DFP relies on the fact that genomic DNA contains hypervariable regions which can be identified using minisatellite or microsatellite probes that enable reliable identification of individuals (Jeffreys et al., 1985a,b). The probes in this method are DNA fragments containing multiple copies of a core sequence. The use of different probes enables the discovery of polymorphic patterns in different animals, and can identify individuals from the same species (Burke and Bruford, 1987; Jeffreys and Morton, 1987; Wetton et al., 1987). The band pattern in DFP is completely specific to an individual and each band is inherited in a Mendelian fashion, making this method a useful tool for tracing pedigree relations and determination of paternity. Identical DFP in members of the same progeny provides proof of genomic identity. The major advantage of this method over other molecular techniques is that no *a priori* knowledge of specific DNA sequences is needed for such proof (Jenneckens et al., 1999). MLR has been interpreted as the *in vitro* analogue of the allograft response test. It consists of measuring the flourishing of leukocytes in response to stimulation by alloantigens (Caspi and Avtalion, 1984; Miller et al., 1986). Both methods depend on activity of the major histocompatibility complex (MHC) that controls the production of strong transplantation antigens and other histocompatibility loci coding for class II antigens involved in the humoral immune response.

Heterozygous and homozygous clones were obtained in a number of fish species (e.g., Komen and Richter, 1990; Komen et al., 1991; Quillet et al., 1991; Bongers et al., 1998; Hussain et al., 1998; Sarder et al., 1999). DFP and skin grafting were used in some of these studies to confirm genetic uniformity. Caspi and Avtalion (1984) have shown the efficiency of MLR in identifying differences among individuals of the Dor-70 line of common carp (*Cyprinus carpio* L.) by using both one way and two way MLR. Both methods were used in the present study to investigate putative heterozygous and homozygous clones of common carp

obtained by induced gynogenesis. The aims of this study were to confirm the genetic uniformity among progeny and to prove that no paternal DNA was transmitted during gynogenetic reproduction.

Materials and Methods

Creation of clones. The fish were bred at the Fish and Aquaculture Research Station, Dor. Preliminary experiments started in 1992, and the two heterozygous and one homozygous clones were obtained in 1994. Standard methods of fish rearing were used. Samples from these clones were taken to Bar Ilan University, Tel Aviv, where they were maintained as described by Rosenberg-Wiser and Avtalion (1982). The experimental fish were produced from the Dor-70 common carp, the most important commercial line of Israeli common carp (Wohlfarth et al., 1980).

The sequence for producing heterozygous and homozygous clones is presented in Fig. 1. The parents of the three clones were gynogenetic females (F_1) obtained from regular common carp females (P) by induced diploid mitotic gynogenesis (Cherfas et al., 1993). Due to the cytological peculiarities of mitotic induced gynogenesis, the F_1 generation (mitotic gynogenetic progeny) was expected to segregate for maternal genes, but each mitogynogenetic individual was expected to be fully homozygous. Further, the gynogenetic generation should be all-female due to female XX homogamety.

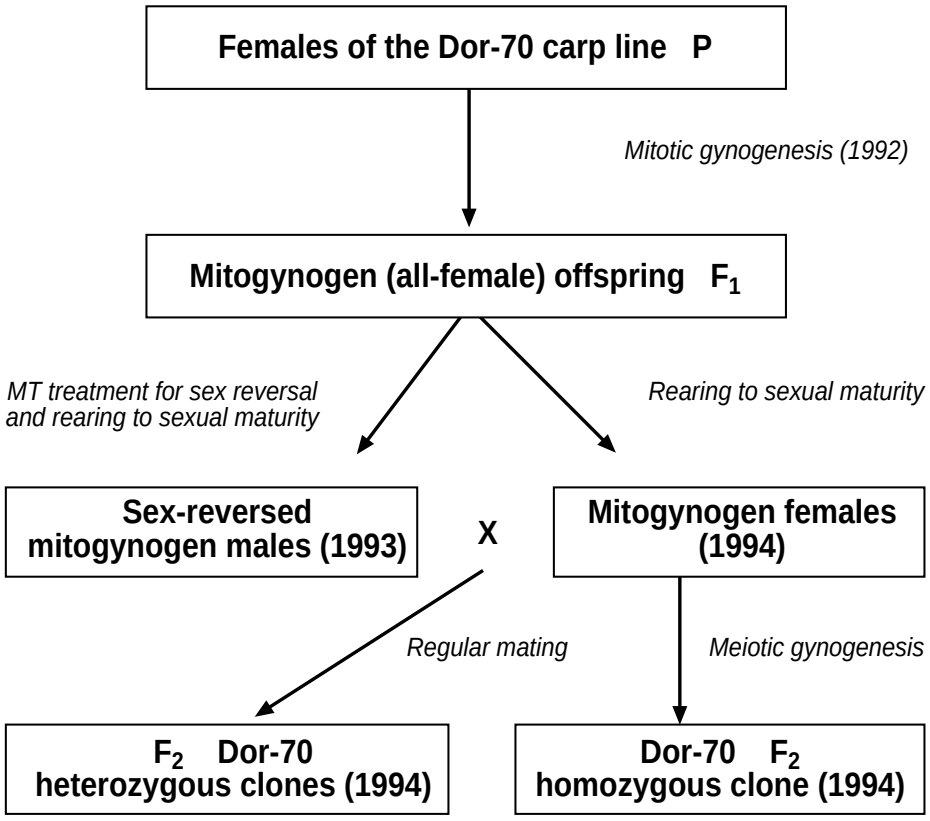

Fig. 1. Production of heterozygous and homozygous clones of the Dor-70 carp line.

Some of the mitogynogenetic fish were subjected to hormonal treatment to induce phenotypic sex inversion (Gomelsky et al., 1994), i.e., sex-inversed gynogenetic males. The heterozygous clones were obtained from two mitogynogenetic females and two (sex-inversed) males through regular crossing after rearing to maturity. Segregation in the F_1 generation provided the heterozygosity in the F_2 generation. The homozygous clone was obtained from the same mitogynogenetic female used to produce clone 1, through induction of meiotic gynogenesis. Genetically inactivated sperm of regular common carp males was used to induce the gynogenetic development (Cherfas et al., 1993). Due to the full homozygosity of the F_1 mitogynogenetic females, no segregation was expected in the meiogynogenetic progeny. Contrary to the fish from F_2 heterozygous clones, the meiogynogenetic fish were expected to be fully homozygous because they possessed only their mother's genotype.

DNA fingerprinting. Blood samples were taken from the founders of the heterozygous clones and from random samples of the mitogynogen females, heterozygous clones and homozygous clone. The bleeding was not sterile, and the syringe was rinsed with a solution to prevent coagulation (0.48 g citric acid, 1.32 g sodium citrate and 1.47 g glucose, diluted in 100 ml distilled water). Samples of 1 ml blood were placed in Eppendorf vials containing 400 µl of the anti-coagulant, and stored at -70°C. The samples were defrosted and divided into 4 Eppendorf vials before starting the DNA extraction. Red blood cells were lysed by adding 0.05M Tris + 0.001M EDTA solution (at pH 8). The mixture was centrifuged for 5 min at 1500 rpm, and the supernatant was discarded. DNA was extracted according to the technique of Maniatis et al. (1982). Aliquots (20 µg) of each DNA sample were digested with the restriction enzyme Hinf I (Takara) according to the manufacturer's instructions. Digested DNA (10 µg) was fractionated by electrophoresis on 0.8% agarose gel for approximately 48 hours until the 2kb fragment of the molecular weight marker had run 18 cm from the loading line. DNA was fixed to the gel and transferred to Hybond-N+ (fp) nylon membrane (Amersham) according to the manufacturer's instructions. Blot pre-hybridization and hybridization with the R-18.1 probe (Haberfeld and Hillel, 1991) procedures were according to Amersham's instructions. Membranes were wrapped in Saran wrap and exposed to RP2-AGFA X-ray film with an intensifying screen at -70°C for varying periods, producing different band intensities.

Mixed leukocyte reaction. Aseptically collected, heparinized peripheral blood (2 ml withdrawn from the caudal vessel) was diluted 1:5 with MEM (Dulbecco's modified Eagle medium, Gibco, Grand Island, NY). Peripheral blood leukocytes (PBL) were obtained from the diluted blood by density centrifugation (Ficoll Paque, Pharmacia) as described elsewhere (Rosenberg-Wiser and Avtalion, 1982). MLR was performed in triplicates in 0.2 ml cultures on flat-bottom 96-well microculture tissue trays (A/S Nunc, Denmark) as reported by Caspi and Avtalion (1984). Cultures contained a total of $1x10^6$ cells per well, at a 1:1 stimulator:responder ratio. The experiments were carried out in two stages. First, averages of five fish from each clone were challenged among themselves, and against wild-type common carp. In the second stage, three fish from each clone were challenged among themselves and against three fish from another heterozygous clone. Two experimental controls were used for each individual: (a) $1x10^6$ cells/well from the same fish - self-control; and (b) stimulation of leukocytes with PHA, by culturing the PBL with PHA (final concentration 6 µl/ml). The first control was expected to result in no response, unless the fish was sick, while in the second control, high proliferation was expected provided the fish was healthy (stressed fish would not respond at all).

The cells were incubated at 28°C in a fully humidified atmosphere with 5% CO_2. The microculture tissue trays were observed daily under a stereomicroscope to determine the peak proliferation day. On the peak day (days 6-7), H^3-thymidine was added to the medium at a final concentration of 1 µ Ci per well. Eighteen hours later, the cells were collected using an automatic cell harvester (Automash, Microtiter, Switzerland) and the H^3-thymidine uptake was

measured using a liquid β-counter (BETAmatic, liquid scintillation counter, Kontrom). Results are presented as stimulation index (SI) values, calculated by the following equation: SI = 2 cpm/(cpm of control a + cpm of control b).

Whenever self-response (control a) was high but no proliferation was obtained when incubated with PHA (control b), the fish was excluded from the data analysis, assuming it was sick or in stress. Likewise, response values below 1 (for unrelated fish) or above 30 were suspected as abnormal and excluded from the analysis, causing a lower d.f. for some comparisons.

Most of the fish were used for both DNA fingerprinting and MLR, but some died before the MLR experiments were performed. Fish were bled at 4-6 week intervals to avoid stress and anemia.

Statistical analyses of the MLR data were performed using the Student's *t* test.

Results

DNA fingerprinting. A complex band pattern (20-40 bands) was visualized using the R-18 probe (Haberfeld and Hillel, 1991) in Hinf I-digested DNA. Distinct bands were seen only at the top of the fingerprint picture, due to increasing background staining from the top to the bottom. Nevertheless, each heterozygous clone had a unique band pattern. Fragment sizes in each figure were determined using the molecular weight marker λ/Hind III which was included in the gel. Fig. 2 shows the band pattern of the male parent (donor of irradiated sperm used to induce gynogenesis) and mitotic gynogenetic F_1 daughters. None of the examined mitogynogen daughters inherited the unique marked bands of the male parent. These results confirm that no paternal DNA had been transmitted during gynogenesis. Fig. 3 illustrates the band pattern of six mitotic gynogenetic females from the F_1 generation.

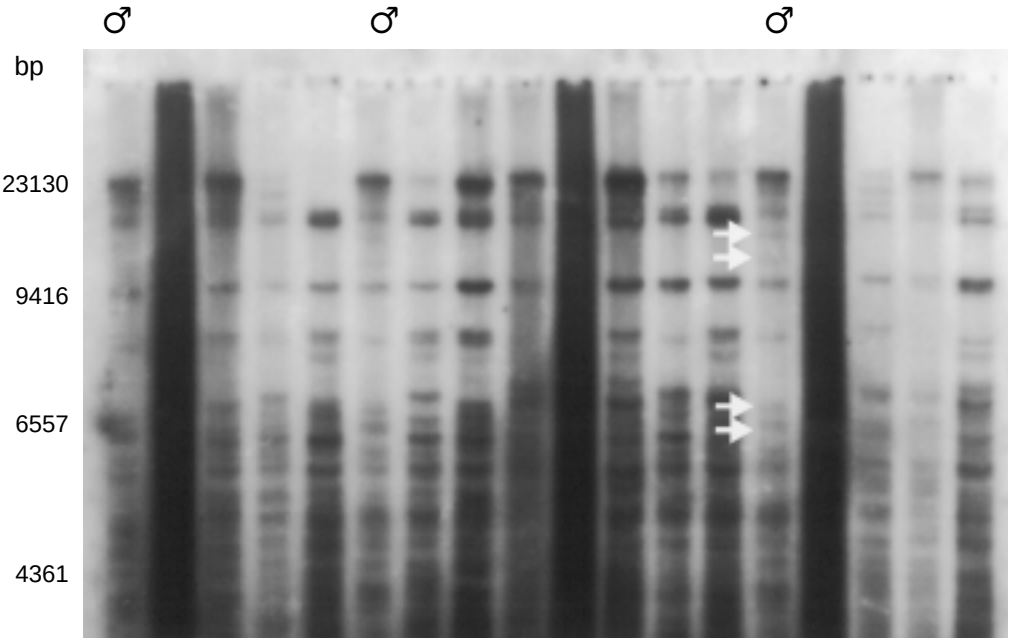

Fig. 2. DNA fingerprinting band patterns of mitogynogen offspring and the male donor of the irradiated sperm used to induce mitotic gynogenesis. The black, non-deciphered columns are the band patterns of the DNA sample of the mother (the sample was, apparently, improperly processed and improper tagging prevented collection of another blood sample from that female.). The arrows indicate unique paternal bands which do not appear in any of the mitogynogen daughters.

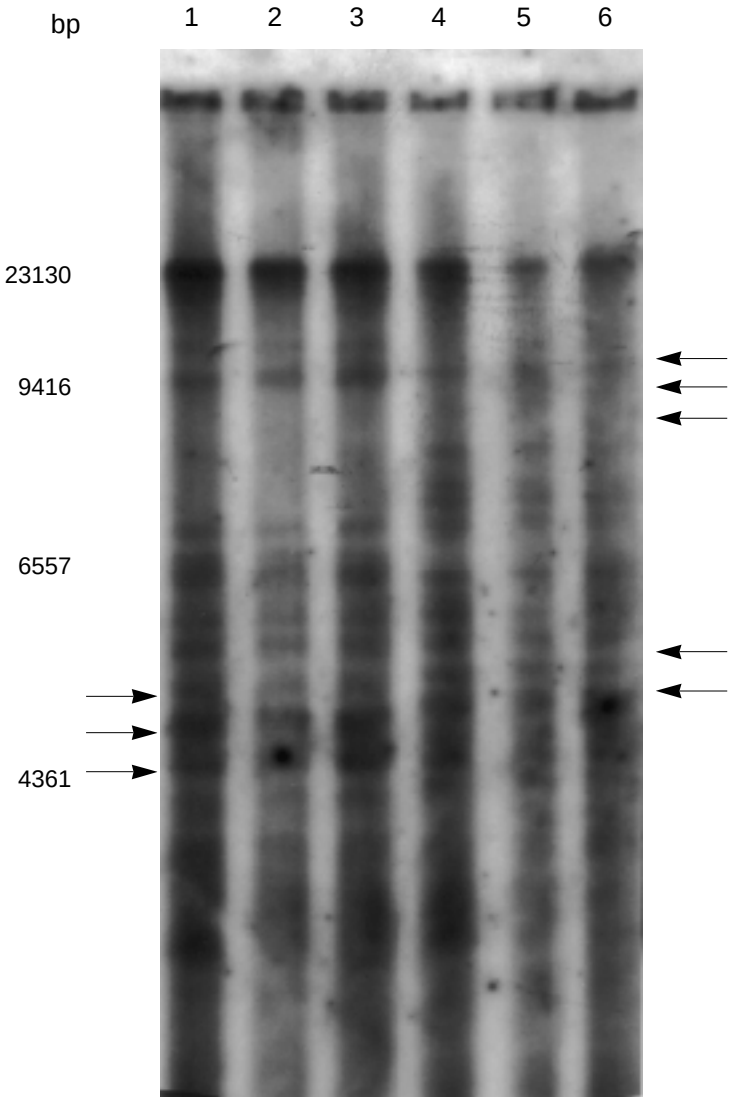

Fig. 3. DNA fingerprinting band patterns in the F_1 generation (mitogynogen offspring). The size markers on the left (23130, 9416, 6557 and 4361 base pairs) were determined using the molecular weight marker λ/Hind III.

Two band patterns were seen (fish 1-3 vs. fish 4-6), showing that the mother was not homozygous. Figs. 4-6 show the highly uniform band patterns of the homozygous clone and the two heterozygous clones.

Mixed leukocyte reaction (MLR) analysis. The optimal harvesting day for MLR response was determined in a preliminary experiment which tested the kinetics of the reaction (data not shown). The peak response was on days 5-8, depending on the combination. The MLR response among individuals within the homozygous and the heterozygous clones was examined as well as the response

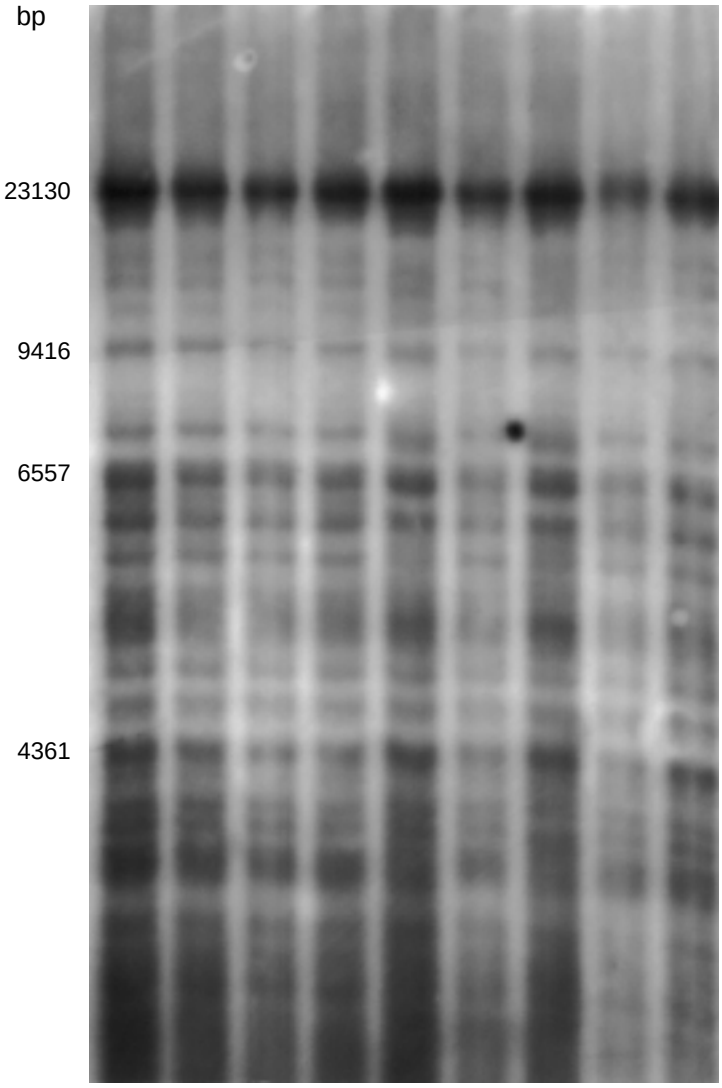

Fig. 4. DNA fingerprinting band patterns in the homozygous clone.

between them and wild type fish (Table 1). The intraclone stimulation indices (SI) were significantly lower than the interline SI values obtained from the responses of the same fish with wild type fish. The MLR responses among individuals within the homozygous and heterozygous clones and between the heterozygous clones were also examined (Table 2). The intra-heterozygous and homozygous clone SI values were significantly lower than the interline SI values obtained from the responses between individuals of the homozygous clone and the two heterozygous clones.

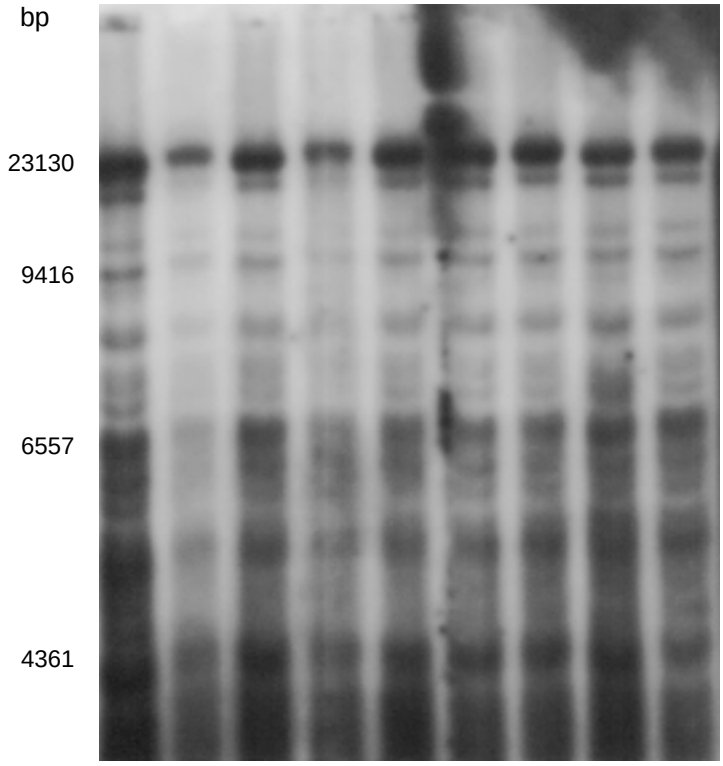

Fig. 5. DNA fingerprinting band patterns in heterozygous clone 1.

Discussion

DNA fingerprinting was used to confirm production of heterozygous and homozygous clones by various authors (e.g., Carter et al., 1991; Harris et al., 1991; Han et al., 1992; Takagi et al., 1993; Volckaert et al., 1994; Heath et al., 1995; Jenneckens et al., 1999; Sarder et al., 1999). Preliminary screening of females from our Dor-70 carp stock revealed a large variation in DFP patterns, to the extent that each had a unique pattern (data not shown). The identical DNA fingerprint pattern of fish within the heterozygous or homozygous clones is evidence of the success in producing heterozygous and homozygous clones from the local common carp in two generations. The identical genomes of fish within the heterozygous and homozygous clones is indirect proof of the homozygosity of their founders (mitotic gynogen males and females), and that no paternal transmission occurred during gynogenesis (mitotic and meiotic). This is in accordance with some earlier investigations, although Carter et al. (1991) and Volckaert et al. (1994) reported on some paternal transmission of markers to putative gynogenes. These unexpected cases were suspected to have resulted from incomplete UV irradiation (Carter et al., 1991) or photoreactivation of UV-irradiated sperm (Ijiri and Egami, 1980; Volckaert et al., 1994).

Immunological response is a way to confirm MHC homology of heterozygous and

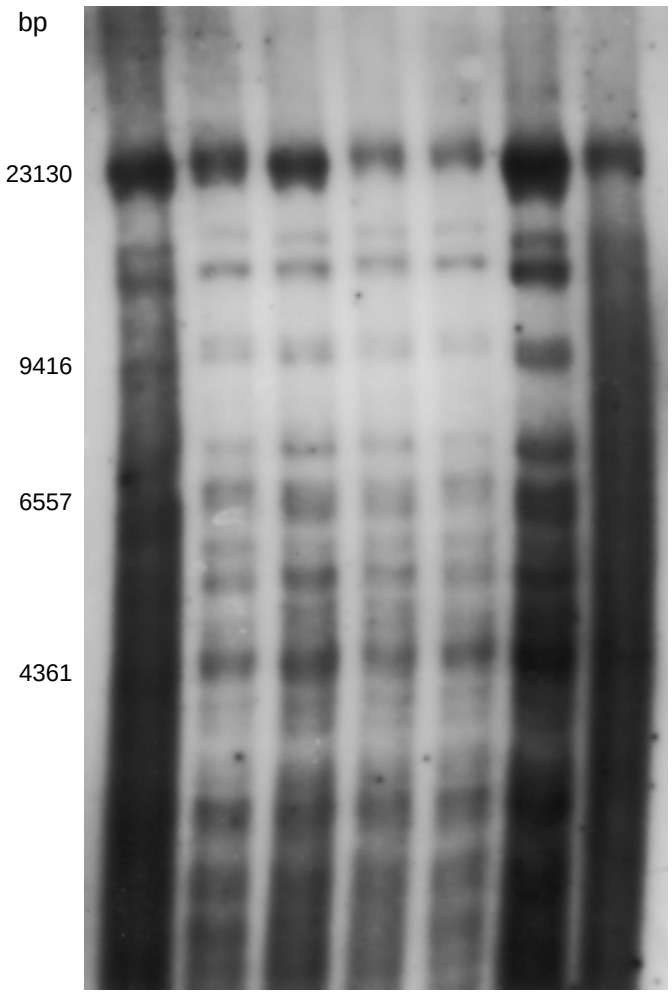

Fig. 6. DNA fingerprinting band patterns in heterozygous clone 2.

homozygous clones, and an indirect way to confirm genome identity. Komen et al. (1990, 1991) used skin grafting to confirm the clonal nature of homozygous clones (homozygous inbred) and heterozygous clones (F_1 strains). Permanent acceptance of allografts was the ultimate proof of success in producing homozygous and heterozygous clones in their studies. MLR has been interpreted as an *in vitro* analogue of allograft response (Caspi and Avtalion, 1984). Very low response in MLR (SI smaller or equal to 1) is considered identical to acceptance of allografts.

The major difficulty with interpretation of MLR data is the variability in magnitude and kinetics of allogeneic and autologous responses (Caspi and Avtalion, 1984; Stet and Egberts, 1991). To overcome this, we used the average responses of replicate fish from each clone and not single pair comparisons.

The MLR response (SI values) obtained among fish within each clone in our study was around 1 (the lowest average was 0.65 and the highest 1.25). These values are considered indicative of genetic uniformity. Stet and Egberts (1991) reported that, within a group of

Table 1. Average (±S.D.) mixed leukocyte reaction (MLR) stimulation index (SI) among five individuals of the homozygous clone (PL), five individuals of each heterozygous clone (Cl-1 and Cl-2), and between them and a wild type (WT) carp.

	SI day 6	*SI day 7*		*SI day 6*	*SI day 7*	*Comparison day 6*	*Comparison day 7*
PL vs. PL	0.65 (0.30)	0.78 (0.31)	PL vs. WT	1.49 (0.28)	2.87 (1.51)	$t_{(d.f. =13)}$= 5.2; p=0.0002	$t_{(d.f. =13)}$= 4.4; p=0.0008
Cl-1 vs. Cl-1	0.95 (0.49)	0.99 (0.57)	Cl-1 vs. WT	5.56 (3.02)	10.13 (8.83)	$t_{(d.f. =13)}$= 4.9; p=0.0003	$t_{(d.f. =13)}$= 3.4; p=0.0048
Cl-2 vs. Cl-2	1.12 (0.65)	-	Cl-2 vs. WT	7.02 (3.01)	-	$t_{(d.f. =13)}$= 6.1; p<0.0001	-

Table 2. Average (±S.D.) MLR stimulation index (SI) among three individuals of the homozygous clone (PL), three individuals of each heterozygous clone (Cl-1 and Cl-2) and between them.

	SI day 6	*SI day 7*		*SI day 6*	*SI day 7*	*Comparison day 6*	*Comparison day 7*
PL or Cl-1	0.75 (0.49)	1.24 (0.82)	PL vs. Cl-1	6.71 (3.14)	12.04 (6.38)	$t_{(d.f. =8)}$= 4.1; p=0.0035	$t_{(d.f. =12)}$= 3.8; p=0.0025
PL or Cl-2	-	0.99 (0.49)	PL vs. Cl-2	-	10.10 (8.31)	-	$t_{(d.f. =11)}$= 2.7; p=0.022
Cl-1 or Cl-2	1.25 (0.74)	0.98 (0.48)	Cl-1 vs. Cl-2	3.88 (1.08)	4.74 (1.19)	$t_{(d.f. =13)}$= 5.2; p=0.0002	$t_{(d.f. =13)}$= 7.3; p<0.0001

second generation gynogenetic siblings, MLR reactions were minimal. However, they did not show SI values, so comparisons with our results cannot be made. On the other hand, the responses of our fish with wild type common carp were strong. SI values were above one (1.49-10.13), 2-8 fold higher than the responses within the heterozygous or homozygous clones. The responses between the heterozygous and homozygous clones were also strong and significant; SI values were 3.88 to 14.99. These results are in general agreement with those of Van Muiswinkel et al. (1986) and Komen et al. (1990, 1991), although the response of the homozygous clone against the wild type carp was weaker than those of the heterozygous clones.

Successful development of homozygous and heterozygous clones of the Dor-70 carp was proven using two independent and complementary methods. The founders of the heterozygous clones (mitogynogen females) must have been genetically homozygous since no band pattern variability was observed in DFP and no MLR response was observed among individuals in either heterozygous clone.

Acknowledgments

This research was supported by research grant no. IS-1612-89 from BARD, the United States-Israel Binational Agricultural Research and Development Fund. Thanks are due to Prof. Joseph Hillel of the Faculty of Agricultural, Food and Environmental Quality Sciences, The Hebrew University of Jerusalem, for allowing us to use the R-18 probe developed in his lab. The authors thank Dr. Sara Levkovitch and Dr. Ronit Zilberman of the Department of Zoology, Tel Aviv University, and Mrs. Ruth Segal and Dr. Bracha Timan of the Bar Ilan University fish immunology laboratory for their assistance.

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