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ESTIMATION OF NUCLEAR DNA CONTENT BY FLOW CYTOMETRY IN FISHES OF THE GENUS *XIPHOPHORUS*

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Abstract—1. By use of flow cytometry we measured nuclear DNA content in cells from 16 stocks representing 9 species of the genus *Xiphophorus*.

2. Significant differences were detected between certain stocks and species with respect to DNA content.

3. Male–female differences were apparent in 5 of 7 stocks in which males and females were studied.

4. Estimation of nuclear DNA content is of potential significance in connection with the genetics of sex determination and the study of taxonomic relationships.

INTRODUCTION

The amount of nuclear DNA, i.e. genomic size, can be estimated by flow cytometry. Cells are stained with a DNA-specific fluorochrome and are analyzed individually for fluorescence, which is directly proportional to nuclear DNA content. Accordingly, DNA content has been studied by flow cytometry in mammals (Sherwood and Patton, 1982; Elias *et al.*, 1988), birds (Vindeløv *et al.*, 1983), reptiles (Bickham *et al.*, 1985), amphibians (Tank *et al.*, 1987) and fishes (Thorgaard *et al.*, 1982). Within the fishes, flow cytometry has been used to measure DNA in a variety of cell types such as erythrocytes and sperm (Benfey *et al.*, 1986; Allen *et al.*, 1986) and in cells from liver, kidney and adipose fin (Utter *et al.*, 1983).

By application of this method, Johnson *et al.* (1987) identified differences in nuclear DNA content between (and within) several species of salmonids. We therefore asked whether the method could be used to identify differences in nuclear DNA content in nine species of the genus, *Xiphophorus*, in which the genetics has been well-studied (Kallman *et al.*, 1973; Kallman, 1974, 1984).

Fish eythrocytes were stained and analyzed together with an internal reference—erythrocytes of the chicken, *Gallus domesticus*—and the results were expressed as picograms of DNA relative to the DNA content of fresh human leukocytes. We detected differences between species, differences between certain stocks within species, and in certain groups, differences between male and female.

MATERIALS AND METHODS

Fishes

The fish used in the study were organized by stock, species and sex (Table 1) and were shipped by air from the New York Aquarium to Memphis, where the groups were maintained in separate aerated aquaria for one week. Scissors were used to remove the caudal fin of each fish, and small volumes of blood were collected into plastic tubes containing 150 μl of phosphate-buffered saline (PBS). The samples were coded and immediately refrigerated, and were then analyzed in random order over a four-day period.

Sample preparation

The erythrocytes (RBC) from these fishes were mixed with RBC from chicken 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. After mixing, the samples were filtered through a 20 micron nylon mesh, and analyzed by flow cytometry within 15 min. The chicken served as an internal standard to correct for transient fluctuations in laser beam intensity, changes in sample flow characteristics, and other factors that can cause variations in DNA readings. Erythrocytes from a male chicken were prepared for use as a standard by dilution of whole blood in PBS containing a final concentration of 8% dimethyl sulfoxide. Cell suspensions were aliquoted, and individual vials were frozen in liquid nitrogen and then stored at −80°C. Each day, similarly-cryopreserved aliquots of RBC from channel catfish (*Ictalurus punctatus*) and female mosquitofish (*Gambusia affinis*) were thawed in a 37°C water bath with the chicken RBC. These standards were compared as a mixture, with fresh leukocytes from males of the domestic pig (*Sus scrofa*) and the human, in order to validate the DNA content of the chicken standard.

Flow cytometry

Analyses were performed by use of a Coulter EPICS V flow cytometer calibrated with a mixture of normal porcine and human leukocytes, and with fluorescent beads. The argon-ion laser was set at 488 nm and 300 mW. Optical filters consisted of two 515 nm long-pass barriers, a 560 nm dichroic splitting filter and a 570 nm long-pass filter placed in front of the photomultiplier tube. Individual nuclei (particles) of lysed RBC were analyzed at a rate of 100–200 particles per second. The fluorescence of each particle was converted to an analog signal that was transmitted to a computer where it was digitized and used to generate pulse-height histograms. Histograms representing at least 30,000 cells were generated with *Xiphophorus* and chicken G_0–G_1 populations placed approximately at channels 125 and 200, respectively, for greatest resolution and accuracy.

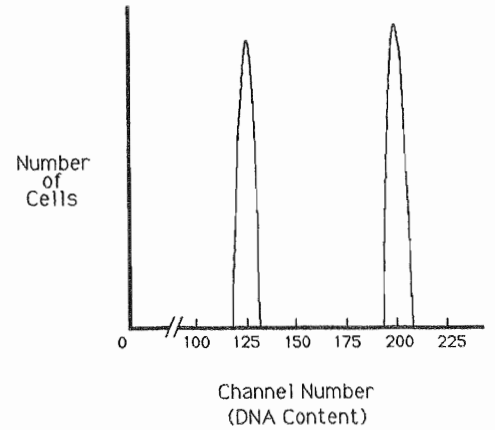

Fig. 1. Graphic representation of a typical DNA fluorescence histogram showing *Xiphophorus* erythrocytes (channel 125) and chicken erythrocytes used as an internal standard (channel 200). Nuclear DNA content is directly proportional to fluorescence intensity, expressed here as channel numbers.

Analysis of data

The Coulter PARA 1 program (Coulter Electronics, Hialeah, Florida) was used to calculate, to four decimal places, the fractional mode channel (FMC) of each histogram peak. Nuclear DNA content from *Xiphophorus* fishes was expressed as pg DNA per diploid cell in relation to an assigned value of 7.0 pg DNA per cell for fresh human leukocytes (from Shapiro, 1976). *Xiphophorus* values were calculated by the formula: DNA content $= (A/B)(C/D) \times 7.0$, where A, B, C and D are FMC values, and B and C, respectively, are the values for the chicken internal standard run simultaneously with the *Xiphophorus* being analyzed (A), or fresh human leukocytes (D). With respect to DNA content, chicken cells are closer to *Xiphophorus* cells than human cells are, and therefore provide a better internal reference standard (Vindeløv *et al.*, 1983). The human leukocytes used as references in the present study were collected from a normal male (RWC) who has provided cells for analysis generally on a weekly basis for the last 7 years. These cells have been compared with cells from hundreds of human subjects. Because they are well-studied and are routinely available from the same donor, these cells could be used in conjunction with chicken cells to provide a consistent system for calculation of DNA mass.

Apparently, *Xiphophorus* RBC take up less stain than is taken up by other cells in the fish; this sometimes generated the appearance of a second peak in *Xiphophorus*, presumably because of release of nuclei from tissues damaged during removal of the caudal fin. Criteria for inclusion of histograms in the data analysis were: (a) RBC fluorescence peak coefficient of variation (calculated at 50% maximum height) less than 4%; and (b) no overlap between RBC and non-RBC peaks large enough to effect changes in FMC. Statistical significance was determined by the Mann–Whitney U test and the Kruskal–Wallis test.

RESULTS

A graphic representation of a typical histogram showing the fluorescence distribution in erythrocytes from *Xiphophorus*, and chicken is shown in Fig. 1. In this system, intensity of fluorescence was directly proportional to DNA content. Thus, cells from chicken—used as an internal standard—contained more nuclear DNA than did cells of the *Xiphophorus* species studied.

Relative fluorescence in cells from male and/or female in 16 stocks from 9 species of Xiphophorus is presented in Table 1. The relative fluorescence is expressed as the ratio of FMC in fish cells to FMC in chicken cells assayed simultaneously. Note that the value of 0.6359 ± 0.0009 (S.E.), found in 3 females of *X. helleri* was the maximum, whereas the values of 0.6057 ± 0.0016 and 0.6054 found in 6 males of *X. andersi* and a single female of *X. pygmaeus*, respectively, were the minimum values. Relative fluorescence data are transformed in Table 2 to give pg DNA per cell; the data are ranked by stock (pedigree number) from highest to lowest (the data were ranked by four decimal places and reported as two decimal places). Statistically-significant differences were noted between species (Kruskal–Wallis, $P < 0.001$); and in certain stocks such as *X. alvarezi* 5508, and *X. maculatus* 5614, 5517, $163A^{73}$, between males and females (Mann–Whitney, $P < 0.05$). Male–female differences were also evident for *X. maculatus* stock 30^{80}, but in this case the male had more DNA. This could be due to confounding of the erythrocyte DNA peak by values more properly attributed to cells of a solid tissue such as muscle or skin (see Discussion).

Table 1. DNA content in nine species of *Xiphophorus*

Species	Pedigree	Relative fluorescence* Male	Female
alvarezi	5508	0.6166 ± 0.0021 (5)†	0.6239 ± 0.0026 (4)
andersi	5519	—	0.6071 ± 0.0022 (6)
	5491	0.6057 ± 0.0016 (6)	—
cortezi	5593	0.6273 (1)	—
	5561	0.6288 ± 0.0017 (3)	—
couchianus	Xc^{43}	0.6124 ± 0.0012 (2)	0.6131 ± 0.0014 (6)
helleri	5457	—	0.6359 ± 0.0009 (3)
	5426	0.6258 ± 0.0013 (6)	—
maculatus	5614	0.6072 ± 0.0024 (4)	0.6187 ± 0.0009 (3)
	5517	0.6134 ± 0.0017 (4)	0.6178 ± 0.0008 (4)
	$163A^{73}$	0.6132 ± 0.0009 (4)	0.6171 ± 0.0012 (5)
	30^{80}	0.6272 ± 0.0005 (2)	0.6153 ± 0.0013 (5)
montezumae	5631	0.6213 ± 0.0011 (5)	—
meyersi	5647	0.6118 ± 0.0024 (5)	0.6112 ± 0.0038 (6)
pygmaeus	5509	0.6077 ± 0.0040 (2)	0.6054 (1)
	5421	—	0.6065 ± 0.0017 (3)

*Expressed as a ratio of the *Xiphophorus* fractional mode channel divided by the fractional mode channel of the chicken internal reference.
†Mean ± SE of scores from (n) fish.

Table 2. Picograms of nuclear DNA in *Xiphophorus*

Female					Male				
Species	Pedigree*	Sex-determining mechanism	*n*	DNA†	Species	Pedigree*	Sex-determining mechanism	*n*	DNA†
helleri	5457	WY	3	1.56 ± 0.00	*cortezi*	5561	XY	3	1.54 ± 0.01
alvarezi	5508	WY	4	1.53 ± 0.01	*maculatus*	30^{86}	XY	2	1.54 ± 0.00
maculatus	5614	WY	3	1.52 ± 0.01	*helleri*	5426	YY	6	1.53 ± 0.01
maculatus	5517	WY	4	1.51 ± 0.00	*cortezi*	5593	XY	1	1.53
maculatus	$163A^{73}$	XX	5	1.51 ± 0.01	*montezumae*	5631	XY	5	1.52 ± 0.01
maculatus	30^{80}	XX	5	1.51 ± 0.01	*alvarezi*	5508	YY	5	1.51 ± 0.01
couchianus	Xc^{43}	XX	6	1.50 ± 0.01	*maculatus*	5517	YY	4	1.50 ± 0.01
meyersi	5647	?	6	1.49 ± 0.02	*maculatus*	$163A^{73}$	XY	4	1.50 ± 0.00
andersi	5519	XX	6	1.49 ± 0.01	*couchianus*	Xc^{43}	XY	2	1.50 ± 0.01
pygmaeus	5421	XX	3	1.49 ± 0.01	*meyersi*	5647	?	5	1.50 ± 0.01
pygmaeus	5509	XX	1	1.48	*pygmaeus*	5509	XY	2	1.49 ± 0.01
					maculatus	5614	YY	4	1.49 ± 0.01
					andersi	5491	XY	6	1.48 ± 0.01

*Pedigree numbers assigned at New York Aquarium.
†Estimated mean picograms ± SD.

Females of four of the stocks analyzed (three species) have the WY mechanism of sex determination (Kallman, 1984; Kallman and Bao, 1987). The DNA readings from these females had higher values than did DNA from XX females. The same was not true for males with the corresponding YY mechanism.

DISCUSSION

The results of the studies reviewed here indicate that flow cytometry is a useful means of estimating total nuclear DNA in fishes. (See also Johnson *et al.*, 1987.) Evidently the method is sensitive enough to resolve differences on the order of 0.02 pg, representing about 2×10^7 base pairs (see Gold and Amemiya, 1987).

Among the nine species of *Xiphophorus* studied, comprising 16 stocks, we observed differences of 0.08 pg in erythrocyte DNA, from the stock with the most DNA [*X. helleri* 5457 females, 1.56 ± 0.00 (S.D.)] to the stocks with the least (*X. pygmaeus* 5509 females, 1.48; and *X. andersi* 5491 males, 1.48 ± 0.01). The mean value for males and females of all stocks studied was 1.51 ± 0.02 pg, which compares favorably with the values of 1.9, 1.6 and 1.4 pg for *X. helleri* and 1.9 pg for *X. maculatus* (cited in Shapiro, 1976).

As noted above, there were differences in the amount of DNA measured in erythrocytes and in the amount measured in cells from solid tissues. Contamination of erythrocyte samples with fragments of skin and muscle during blood collection, could account for the high readings obtained for the two *X. maculatus* males from stock 30^{80} (compare with readings for males from the same species, stocks 5517 and $163A^{73}$ in Table 2). In general, members of different stocks within the same species had similar amounts of DNA. Between stocks, the differences in DNA values were continuous and overlapping. A similar finding was reported for 20 cyprinid species on the basis of a microspectrophotometric analysis for Feulgen-stained erythrocyte nuclei (Gold and Amemiya, 1987).

Sex differences were detected in 5 of 7 stocks for which males and females were available. This was unexpected because heteromorphic sex chromosomes have not been described in any of these species. Nevertheless male and female heterogamety each have been described in this genus, and in fact, homozygous and heterozygous males *and* females can occur in the same population, in *X. maculatus*. In this species, the "platyfish", there is a modified X chromosome called the "W", which blocks the male-determining function of the Y. Three kinds of females are commonly found: XX, WX and WY; and two kinds of males: XY and YY. Thus the platyfish may represent an evolutionary stage at which female heterogamety emerges as a result of a single modification in a male-heterogametic system (Kallman, 1984).

We have noted that cells from females of the WY species or stocks tested, contain more DNA than do cells from the other females tested. So the question arises whether this may be related to a structural modification in the W chromosome involving gene amplification or heterochromatinization, or whether it may reflect differences between species or species groups. If the first interpretation is correct, females of *X. meyersi*, which had DNA values below those for WY females, might be homogametic (XX).

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