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DNA Content in Eurasian Sturgeon Species Determined by Flow Cytometry

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The nuclear DNA content in 10 species of chondrostean fishes was measured by flow cytometry. The sterlet *Acipenser ruthenus* blood cells were used as an internal standard. The sterlet DNA content was calculated on the basis of comparison with the *Xenopus laevis* blood cells, 2C = 6.30 pg. In the tetraploid *A. ruthenus* and *A. stellatus* the DNA content comprises 3.74 pg/nucleus and is practically invariant; in *Huso dauricus* it is almost the same, 3.74–3.81 pg; and in *A. nudiventris* it is a little higher, 3.88–4.04 pg. In the oldest chondrostean, *Pseudoscaphirhynchus kaufmanni*, the nuclear DNA content is slightly lower, 2C = 3.46–3.48 pg, and in the American paddlefish *Polyodon spathula* it is lower still, 3.17 pg. In two octoploid sturgeons, *A. baeri* and *A. gueldenstaedti*, the DNA content is twice as high as that of the sterlet, 8.29–8.31 and 7.86–7.88 pg, respectively; a very similar amount, 8.24–8.42 pg, was determined in the hybrid *Huso huso* × *A. ruthenus*. In the Sakhalin sturgeon, *A. medirostris* (=*A. mikadoi*), the DNA content is two times higher than in the octoploids, 13.93–14.73 pg; therefore its ploidy may be 16n and the number of chromosomes could be 500. © 1993 Wiley-Liss, Inc.

Key terms: Flow cytometry, nuclear DNA content, chondrostean fishes

Flow cytometry is one of the most sensitive techniques used for quantifying cellular DNA (reviews in 14,52,62) and has been used to determine the nuclear DNA content of many animal species, including vertebrates from fishes to mammals (11,12,36,37,40,42,53, 57,58,60), and mollusks (1,19). It was also used for determination of cell or sperm ploidy in fishes, amphibians, and reptiles (1,2,6,7,21,22,35,61), sex identification in birds (45), as well as for investigation of genome elimination of one of the parents in hybridogenetic frogs (10,66) and hybrid fishes (27). In the present study, we have used flow cytometry technique to measure the DNA content in species of one of the most ancient fish groups, the chondrosteans, inhabiting the former USSR territory. Recently the same measurements were done on the American species (9).

The chondrosteans are interesting fishes not only because they are ancient and inhabit two continents, Eurasia and North America, but also because the species investigated so far are tetraploids (4n = 120) or octoploids (8n = 240), a very unusual characteristic in vertebrates (8). The data on ploidy and DNA content determined before by microspectrophotometry of Feulgen-stained blood cell nuclei (mainly European species) and by flow cytometry (the American species) is given in Table 1. Our new results show a two-fold difference in the DNA content between tetra- and octoploid sturgeon species. Moreover, it appeared that in one species, the Sakhalin sturgeon *Acipenser medirostris*, the nuclear DNA content is twice that found in the octoploids. If the chromosome number in this species is proportionally increased, it means that the Sakhalin sturgeon has the greatest diploid number among vertebrates, about 500 chromosomes.

MATERIALS AND METHODS

Fishes

Fishes from the unique collection of sturgeon species maintained in the Moscow Aquarium were used. The names of the species, areas where they were caught, and the number of individuals studied by us are listed in the Table 2. It is necessary to mention that two of

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Table 1
Diploid Numbers and DNA Content in Chondrosteans[a]

Species	Chromosome number	2C, pg	Reference
Family Acipenseridae			
Acipenser baeri (Lena River, Siberia)	248 ± 5	—	64
A. brevirostrum (North America)	—	13.08[b]	9
A. fluvescens (North America)	—	8.90[b]	9
A. gueldenstaedti (Volga River, European Russia)	250 ± 8	—	4, 8, 63
A. medirostris (North America)	—	8.82[b]	9
A. naccarii (Italy)	239 ± 7	—	24
	—	5.7–6.3[c]	23
A. nudiventris (Aral Sea)	118 ± 2	—	3, 63
A. oxyrhynchus desotoi (North America)	—	4.55[b]	9
A. ruthenus (different European populations)	116 ± 4	—	25
	118 ± 2	—	8, 63
	118 ± 3	—	48
	118 ± 3	—	5
A. schrencki (Amur River)	(240±)[d]	—	50
A. sinensis (China)	264±	—	67
A. stellatus (Volga River, European Russia)	118 ± 2	—	8, 63
A. sturio (Italy)	116 ± 4	—	24
	—	3.6[c]	23
	—	3.2	43
A. transmontanus (North America)	(230)[e]	—	29
	—	10.6[c]	30
	—	9.56[b]	9
Huso dauricus (Amur River)	(120)[d]	—	50
H. huso (different European populations)	116 ± 4	—	24
	—	3.6[c]	23
	118 ± 2	—	8, 63
	118 ± 3	—	5
Scaphirhynchus platorhynchus (North America)	112±	3.6[c]	47
	—	4.73[b]	9
Family Polyodontidae			
Polyodon spathula (North America)	120	—	20
	—	3.9[b]	57
	—	4.89[b]	9

[a]All species investigated karyologically so far are listed; the DNA content values for all species studied before are given.
[b]Determined by flow cytometry.
[c]Determined by microdensitometry of Feulgen-stained nuclei.
[d]Only macrochromosomes were counted precisely.
[e]Chromosome No. was determined in cell cultures: the modal 2n in a spleen cell line was 219, and in a heart cell line, 237–243.

these species, the Sakhalin sturgeon *Acipenser medirostris* and especially the large Amu-Dar shovelnose *Pseudoscaphirhynchus kaufmanni,* are endangered, while the Aral ship, *A. nudiventris,* has already become extinct in the wild and only a few individuals are still living in the Aquarium.

Sample Preparation

From each fish 0.5 ml of whole blood was taken by a heparinized syringe and suspended in 25 ml of 3.8% sodium citrate with 0.02% EDTA (60). The suspensions were stored on ice during transportation from the Aquarium to the laboratory. Then the blood cells were pelleted by centrifugation at 600*g* for 5 minutes and resuspended in 2 ml of 0.01 M Tris pH 7.2, containing 5 mM EDTA and 50 mM KCl. A part of cell suspensions were then used for immediate staining with the remainder being refrigerated (4°C) after fixation with 45–50% ethanol (32). The samples measured contained 0.5 ml of lysis-staining buffer (the same Tris-buffer with 0.1% Triton X-100 and 30 μg/ml propidium iodide) and 50 μl of washed cell suspension. Several microliters of the sterlet *Acipenser ruthenus* cell suspension was added as an internal DNA standard. Measurements were made after 15 minutes incubation at room temperature. In a few cases (ship, starred sturgeon) the measurements were repeated with material fixed in 45% ethanol after keeping it at 4°C for 2–3 days.

Table 2
DNA Content Measurements and Descriptive Statistics in Chondrostean Species Studied[a]

Species	Chromosome number[b]	N	2C (pg) 2C	SD	CV(%)	Range (pg)
Family Acipenseridae						
Acipenser ruthenus (sterlet, Volga River)	118	2	3.74	0.14	3.78	3.74
A. baeri (Siberian sturgeon, Lena River)	248	2	8.31 8.29	0.18 0.15	2.19 1.78	8.29–8.31
A. gueldenstaedti (Russian sturgeon, Volga River)	250	3	7.88 7.86 7.88	0.20 0.15 0.15	2.49 1.94 1.94	7.86–7.88
A. medirostris (Sakhalin sturgeon, Far East)	—	4	13.93 14.15 14.51 14.73	0.89 0.90 0.82 0.90	6.40 6.40 5.70 6.14	13.93–14.73
A. nudiventris (Aral ship, Aral Sea)	118	3	4.04 3.89 3.88	0.10 0.11 0.13	3.01 2.91 3.78	3.88–4.04
A. stellatus (starred sturgeon, Volga River)	118	2	3.74 3.74	0.11 0.14	2.97 3.79	3.74
Huso dauricus (kaluga, Amur River)	120	2	3.74 3.81	0.13 0.10	3.54 2.74	3.74–3.81
H. huso (beluga, Volga River)	118	3	2.44 2.42 2.45	0.09 0.08 0.08	3.67 3.14 3.46	2.42–2.45
Huso huso × *A. ruthenus*, (bester, an artificial hybrid)	111[c]	2	8.24 8.42	0.12 0.18	1.49 2.21	8.24–8.42
Pseudoscaphirhynchus kaufmanni (large Amu-Dar shovelnose, Amu Darya River)	—	2	3.46 3.48	0.08 0.07	2.45 2.11	3.46–3.48
Family Polyodontidae						
Polyodon spathula (American paddlefish)	120	1	3.17	0.10	3.26	3.17

[a]N, sample size; 2C, nuclear DNA content; SD, standard deviation.
[b]For references, see Table 1.
[c]Data from (5).

Flow Cytometry

DNA analysis was carried out on a EPICS-C flow cytometer (Coulter Electronics, Hialeah, FL) with an argon laser operated at a wavelength of 488 nm at 300 mW in a light-stabilized mode. The emitted light was collected through a 515 nm LWP, a 560 nm dichroic, and 600 nm LWP filters. The values of standard deviations and CVs of peaks were obtained by means of standard programs of the EPICS-C. We collected the fluorescence values of 10,000–50,000 stained nuclei to calculate the DNA content relatively to the internal standard. In the cases when the fluorescence peaks of the sample and standard were not separated enough to be unambiguously discriminated we tried to get information from the differences between the CV values of isolated constituent components and the total peak measured in the experiment.

RESULTS

Measurement Peculiarities

We wish to emphasize two details of measurements. First, when we used the citrate buffer recommended in (60) for the same type of experiments, the peaks in histograms were wider than we had been expecting, taking into account the cytometer calibration using fluorescent microspheres or DNA content measurements of other cell types. We received the best results using not citrate, but Tris-buffer for staining and measurements.

And second, it appeared that the measurements can be obtained on cells fixed in 45% ethanol and kept at 4°C for at least for a few days. In such measurements the peaks in histograms became wider, the CV were increased, but the positions of peaks of experimental and standard samples remained as they were in the case of non-fixed cells. It is interesting that in (42) the authors fixed the nuclei of salamandrids and the control species (*Xenopus laevis*) in ethanol before making their measurements.

We must also mention that in control experiments with RNase present in the staining buffer, the results of measurements were not different from those when it was absent. When we used the cells fixed in 45% ethanol, the RNase treatment was absolutely necessary.

The DNA Content

The DNA amounts in the species investigated are given in Table 2. All data were calculated relative to

the DNA content of the sterlet which was used as an internal standard (Fig. 1b–j). In other words, we added an aliquot of sterlet cell suspension to every sample under investigation, which was stained and analyzed in parallel with the experimental one. In the case of a few samples there were two peaks in histograms obtained (Fig. 1b,e–g), while in the others, when the DNA content of the sterlet and the species under investigation were close, partly discriminated bimodal peaks were seen (Fig. 1c,h). In others, one peak (Fig. 1d,i,j) was observed, with an enlarged (as compared to the experimental sample and the internal standard measured individually) variation coefficient.

The sterlet was selected as an internal standard because in three series of experiments it gave the same invariant results: The DNA fluorescence peak positions were practically the same for different sterlet individuals. The DNA content for the sterlet was calculated from an experiment in which the *Xenopus laevis* blood was used as an internal standard (Fig. 1a). The results obtained from several experiments which utilized microspectrophotometric and biochemical methods were virtually the same (18,26,55), 6.30 ± 0.18 pg per nucleus (26). The DNA content of the sterlet, 3.74 pg, was calculated by using the formula DNA (pg) = 6.30 pg F/S, where F and S are, respectively, the fluorescence of sterlet and *X. laevis* nuclei. The data for the other sturgeon species was calculated according to the same formula, DNA (pg) = 3.74 pg (the sterlet) F/S.

From Table 2 it is clearly seen that the DNA content in three species, the sterlet, starred sturgeon, and kaluga, is almost the same, about 3.7 pg, while in the ship it is a little higher, 3.9–4.0 pg, and in the big Amu-Dar shovelnose, on the contrary, it is a little lower, 3.5 pg. In three beluga specimens the DNA content is 1.5 times lower than in the sterlet. In two sturgeons, Siberian and Russian, it is twice as high as in the sterlet, 8.3 and 7.9 pg, respectively, and in the Sakhalin sturgeon it is increased two-fold as compared with this quantity, 13.9–14.7 pg. In the hybrid bester, the DNA content is twice that of the sterlet, about 8.3 pg.

DISCUSSION

A Problem of the Internal Standard

During our measurements, we tried to minimize the experimental error caused by possible differences in the level of staining (because of some uncertainty in the concentration of the fluorescent dye and/or cells in the samples) by using an internal standard method (54,65).

The DNA content in the standard species, the sterlet, was determined on the basis of comparison with the blood cells of *Xenopus laevis,* a species with well-known DNA content, 2C = 6.30 pg (26). We believe that such a choice of a standard allowed us to receive more exact results than with commonly used chicken erythrocytes (12,36,46,54,56,57,65), human (9,34,57) and swine (57) leukocytes, or mice spleen cells (12,53).

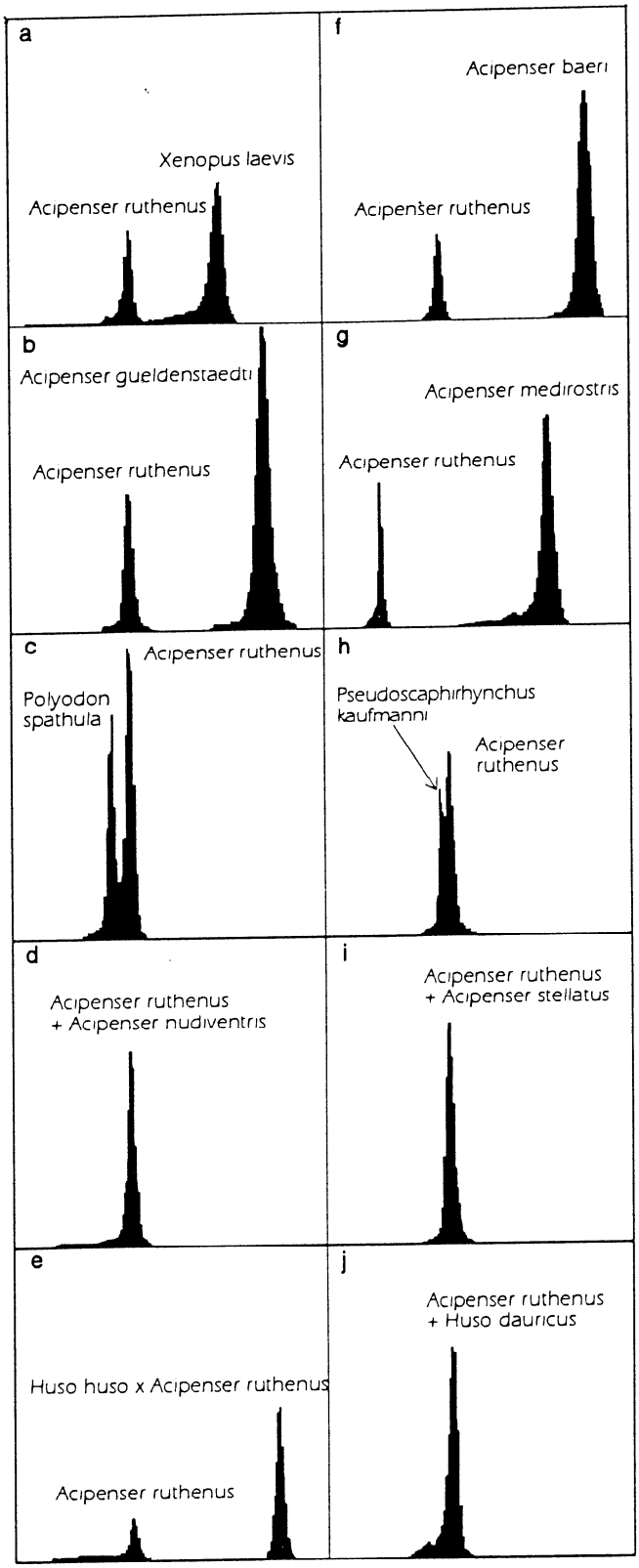

FIG. 1. Histograms of mixtures of different sturgeon species blood samples. The blood of *Acipenser ruthenus* was used as an internal standard. In every histogram the *A. ruthenus* peak occupies channel 100. In the histogram (g) the abscissa scale has been reduced by half.

It is well known that the level of fluorescence signals after the staining of cell nuclei by the DNA-specific dyes depends in general on the following factors (13–15,39): the DNA content, the accessibility of DNA for staining within the chromatin, and the saturation of the chromatin under staining by the dye. As we were interested in the DNA content, we tried to diminish the influence of the other two factors. The use of the internal standard allowed us to minimize and control the influence of staining level, as well as the kinetic effects of the process of staining.

As for the second factor, the accessibility of the DNA in the chromatin of cell nuclei under investigation, we used the standard of the same type of cells and a rather related organism, an amphibian *Xenopus laevis*. Regrettably, we did not have an opportunity to obtain the erythrocytes of a bony fish, the trout, which are frequently used as an internal standard for flow cytometry (33,65). We suppose that the difference in chromatin structure which could be present in cells of different tissues of phylogenetically distant animals, such as fishes and mammals, can influence and even change the results of measurements and turn them to a nonproportional correlation of the DNA content. At least the dependence of the accessibility of different types of chromatin compaction to the dyes is well known (16,17,37,43). That is why the use of chicken or mammalian standards for measurements of the DNA content in cells of fishes (36,56,57) seems incorrect to us. Apparently, the use of nonadequate standards (the swine (57) or human (9) leukocytes) caused the difference in values of the paddlefish *Polyodon spathula* DNA content determined in (57), (9), and by us, 3.90, 4.89, and 3.17 pg, respectively. Possibly, the use of human blood as the internal standard resulted in slightly increased DNA values for the chondrostean species studied in (9) as compared with the previous cytospectrophotometry (Table 1) and our flow cytometry (Table 2) data.

Because of analogous considerations we do not describe the results of experiments with Hoechst 33258: although histograms with small CV values could be obtained with this dye, it is AT-base pair specific (28). This specificity can result in a change in fluorescence signals caused not only by the difference in the DNA content, but also by variations in the AT-content (see, for instance, 36). Using propidium iodide allowed us to diminish the influence of this factor.

The DNA Content in Sturgeons

It is evident from Table 2 that the DNA content per nuclei in the sterlet and starred sturgeon is the same and invariable, 3.74 pg; the kaluga, a representative of the other acipenserid genus, *Huso,* has practically the same value, 3.74–3.81 pg. The large Amu-Dar shovelnose, *Pseudoscaphirhynchus kaufmanni,* which is considered to be the oldest representative of all living chondrosteans, has a slightly decreased DNA content, 3.47 pg/nuclei. The representative of the second chondrostean family, the paddlefish *Polyodon spathula,* has a still lower DNA content, 3.17 pg/nucleus. To our surprise, the beluga specimens investigated had a 1.5 times lower DNA amount (not shown in Fig. 1), 2.44 pg/nucleus, than the sterlet or a representative of the same genus, the kaluga *Huso dauricus,* which has the same chromosome number as the beluga and sterlet, about 120 chromosomes (Table 1). Previous measurements of the DNA content in the erythrocytes of *H. huso* specimens from Italy made by the microspectrophotometry of Feulgen-stained nuclei showed a 1.5 times higher value, 3.6 pg/nuclei (23). Possibly, these data point to some taxonomical difference of beluga forms from these two populations. On the contrary, the Aral ship *Acipenser nudiventris,* has an increased DNA content as compared with the sterlet: 3.88–4.04 pg/nucleus. It is interesting that the preceding cytophotometric measurements gave close results, 3.2–3.6 pg/nucleus for the species we have not studied, *Acipenser sturio,* and *Scaphirhynchus platorhynchus* (Table 1).

The sterlet, ship, starred sturgeon, beluga, kaluga, and paddlefish are ancient tetraploids (8) having about 120 chromosomes (Table 1). Taking into consideration that the large Amu-Dar shovelnose has almost the same DNA content as these species enumerated, one can conclude that it is also a tetraploid with the same chromosome number. The DNA content can be considered as an additional proof of the tetraploidy of the species under consideration: In most diploid teleosts the DNA content is about 2.0 pg/nucleus (31), and only in fishes of tetraploid origin it is two times and more higher, as in salmonids, 4.5–5.7 pg/nucleus (36).

The DNA content in the Russian and Siberian sturgeons is twice that of the sterlet, 7.87 and 8.30 pg/nucleus, respectively (Table 2). This data confirms the octoploid nature of these species (8; Table 1). It is necessary to stress that the genome size differs a little in these octoploid species with the same chromosome number, $2n = 250$. It is not clear if the DNA content in the third octoploid species, the Adriatic sturgeon *Acipenser naccarii,* is really lower (5.7–6.3 pg/nucleus; Table 1) because the lower value could be a result of the cytophotometric method of measurement used in (19). The cause of a high DNA content, 8.24–8.42 pg/nucleus, in the bester individuals (artificial hybrids between the beluga *Huso huso* and the sterlet *Acipenser ruthenus*) investigated by us is not clear. It is known that the basic chromosome number in three generations of such hybrids does not differ from that of parental species but a gradual displacement of karyotypic parameters toward those of the sterlet occurs (5). The latter species has evidently not only invariable DNA content, but also a "dominant" karyotype.

The most unexpected result is that the DNA content in the Sakhalin sturgeon, *Acipenser medirostris,* is twice that of the octoploid species, being 13.93–14.73 pg/nucleus (Table 2). According to this value, the ploidy of the Sakhalin sturgeon should be 16n. This species has not been studied karyologically yet, but on

the basis of DNA content one can propose that its karyotype probably consists of 500 chromosomes. In other words, the Sakhalin sturgeon might have the largest chromosome number in vertebrates.

It is supposed that the same species, the green sturgeon *A. medirostris,* also inhabits the Pacific coast of Canada and the United States (33). According to flow cytometric measurements of Blacklidge and Bidwell (9), the DNA content in the American representatives is 8.82 pg/nucleus. Therefore, the American green sturgeon seems to be an octoploid and perhaps another species, differing from the Sakhalin sturgeon in ploidy. These data support an old point of view that the Asian form of *A. medirostris* is at least a subspecies, *A. m. mikadoi* (41,49), or even a species, *A. mikadoi.*

As for the other American species (not taking into consideration that the values in (9) seem to be a little high), it is evident that only two of them, *Acipenser oxyrhynchus desotoi* and *Scaphirhynchus platorhynchus,* are tetraploids (Table 1), and have DNA contents comparable to that in the tetraploid Eurasian species (Table 2). *A. fluvescens* and the American *A. medirostris* are octoploids, while the ploidy of *A. brevirostrum,* according to Blacklidge and Bidwell (9), is 12C. These authors hypothesize that this species could be formed after a duplication of genome of spontaneous ancestor triploids. Most probably, the ploidy of *A. brevirostrum* is similar to that of the Sakhalin sturgeon and equals 16n. However, it will only be known for sure after cytogenetic analysis. It is not clear if the slightly high DNA amount in the white sturgeon, *A. transmontanus,* 9.46 or 10.6 pg (Table 1), reflects also an increase in ploidy more than 8n. It is interesting that within this species two forms of fishes differing in the genome size have been found: The mean DNA content of the Snake River white sturgeon, 9.115 pg/nucleus, is significantly ($P < .05$) smaller than that of the Columbia River or the San Francisco Bay white sturgeon, 9.587 and 9.548 pg/nucleus, respectively (9). Possibly, these data point to some taxonomic difference between these forms.

On the whole, a conclusion can be made that the investigation of the DNA content in chondrosteans by flow cytometry allowed us to show the existence of a polyploid row, 4n-8n-16n, in these fishes, a situation which is extremely unusual for vertebrates.

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