Fakulta rybářství
a ochrany vod
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Jihočeská univerzita
v Českých Budějovicích
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in České Budějovice

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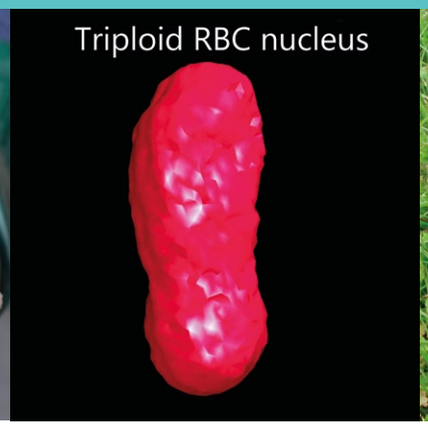

Dmytro Bytyutskyy

Czech Republic, Vodňany, 2014

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CONTENT

CHAPTER 1

GENERAL INTRODUCTION

1.1. INTRODUCTION

Order Acipenseriformes includes 27 fish species (Hochleithner and Gessner, 2006). All existing sturgeon species currently inhabit oceans and rivers of cold- to temperate zones of Northern hemisphere of the planet (Fig. 1). Sturgeons are famously known because of their high quality meat and eggs (black caviar), which are considered as delicacies in many countries. During the last century, sturgeon over fishing in densely populated areas of Europe and America (Ludwig, 2008; Pikitch et al., 2005), constructions of hydropower dams, which blocked the way of natural migration of these fish to spawning areas, as well as environmental contamination caused serious damage to sturgeon populations and led them on the brink of extinction. Nowadays sixteen sturgeon species are considered to be critically endangered and added into the IUCN (Red List of Threatened Species 2013). There are number of international programs and national action plans for reintroduction of individual sturgeon species as well as activities of the World Sturgeon Conservation Society (www.wscs.info) and its various national branches that were initiated in order to save these species (Rosenthal et al., 2011). Moreover, the entire order of Acipenseriformes was involved in 1997 into the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) to ensure that international trade in specimens and products of sturgeons and paddlefishes does not threaten their survival (www.cites.org).

Sturgeon species are interesting not only because of their conservation, wide distribution and economic issues but also because of their evolutionary age, phylogeny and genome evolution due to multiple genome duplication events, as well as of biological characteristics of such ancestral taxa (Ludwig et al., 2001). Last studies on molecular divergence time estimates indicate that Acipenseriformes had diversified by the end of the Carboniferous (~ 300 Ma) (Near and Miya, 2009). There were not many morphological changes happening in these fishes (Gardiner, 1984). From the evolutionary point of view, sturgeons are interesting due to polyploidy origin and possibility of interspecific hybridization in this fishes (Vasil'ev, 2009). Nowadays, due to the large public interest, research on sturgeon biology is constantly increasing, bringing variety of scientific information concerning this species.

1.2. PLOIDY LEVELS IN ACIPENSERIFORMES

Polyploidy shows increases in number of chromosomal complements in cell nuclei. Ploidy types are labelled according to the number of chromosome sets (n) in the nucleus: haploidy (one set; 1n), diploidy (two sets; 2n), triploidy (three sets; 3n), tetraploidy (four sets; 4n) and higher. Order Acipenseriformes shows wide range of ploidy levels, but due to the controversy in scientific circles two ploidy level scales were proposed: a) the 'evolutionary scale' which presumes tetraploid-octaploid-dodecaploid relationships; considered all sturgeon species with ~ 60 chromosomes to be diploids and recently have disappeared, species with ~ 120 chromosomes to be tetraploids (Ohno et al., 1969; Dingerkus and Howell, 1976), species with ~ 250 chromosomes to be octaploids (Birstein and Vasiliev, 1987; Birstein et al., 1997), and species with ~ 360 chromosomes to be dodecaploids; b) the 'recent or functional scale', which presumes diploid-tetraploid-hexaploid relationships; considered all sturgeon species with ~ 120 chromosomes to be diploids (Fontana, 1994; Fontana et al., 1998), species with ~ 250 chromosomes to be tetraploids (Ludwig et al., 2001), and species with ~ 360 chromosomes to be hexaploids (Kim et al., 2005; Fontana et al., 2008b). In accordance with chromosome number, all Acipenseriformes is possible to divide at least on a three discrete groups. The first group includes species with ~ 120 chromosomes, the second

group includes species with ~ 250 chromosomes, and the third one includes species with ~ 360 chromosomes (Fontana et al., 2008b).

Figure 1. *Acipenseriformes distribution. – Acipenser distribution; – Scaphirhynchus distribution; – Polyodon distribution; – Huso distribution; – Pseudoscaphirhynchus distribution.*

There is an assumption that two ploidy level scales appeared due to technical limitations of earliest karyotype studies which did not allow identification of small microchromosomes (Fontana, 2002; Havelka, 2013). Microchromosomes represent about half of sturgeon chromosomes and high number of these small chromosomes cause problem for their exact calculation. On the other hand, there are quite contradictory data on the detection of odd ploidy levels such as penta-and hepta ploidy that do not fit into the 'functional scale' well but likely fit into the 'evolutionary scale'. It is necessary to perform a considerable amount of research in order to get more information about odd- ploidy levels in sturgeons and explain how do they fit into the 'functional scale'. To avoid confusion, only the evolutionary ploidy level model will be referenced in this thesis to represent full diversity of possible ploidy levels in Acipenceriformes fishes.

According to data from Table 1 we can argue that such species as *Polyodon spathula, Acipenser nudiventris, A. oxyrinchus, A. ruthenus, A. stellatus, A. sturio, Huso huso, Scaphirhynchus platorhynchus, Pseudoscaphirhynchus kaufmanni* have ~ 120 chromosomes and ~ 2.4–4.7 pgDNA.nucleus^{-1}. Such species as *Acipenser baerii, A. fulvescens, A. gueldenstaedtii, A. medirostris, A. mikadoi, A. naccarii, A. persicus, A. schrenckii, A. sinensis, A. transmontanus,* have ~ 250 chromosomes and ~ 7.8–10.2 pgDNA.nucleus^{-1}. The third group with ~ 360 chromosomes and ~ 13–13.8 pgDNA.nucleus^{-1}, consists only of *Acipenser brevirostrum.* The highest ploidy level in Asipenseriformes was found in *Acipenser brevirostrum* exhibiting hexadecaploidy (sixteen sets; 16n) (Hardie and Hebert, 2003). Species as, *Psephurus gladius, Scaphirhynchus* and *Pseudoscaphirhynchus* were considered to be tetraploids (Birstein and DeSalle, 1998; McQouwn et al., 2000; Ludwig et al., 2001), wile *A. dabryanus* supposed to be octaploid (Birstein et al., 1997; Zhang et al., 1999). Author's data (Table 1) on species as*, A. persicus, A. medirostris, Huso dauricus, Pseudoscaphirhynchus kaufmanni,* exhibit discrepancies that require further verification.

The entire situation is yet more complicated with capability of sturgeons i) to create autopolyploids *de novo*, i.e. in each generation and ii) to hybridize interspecifically or even intergenerically inside the groups of species of the same ploidy level, as well as between groups of species of different ploidy levels, whilst almost all autopolyploids and some hybrids can be morphologically indistinguishable from pure species.

As polyploidy is an ongoing process, much more investigation is needed for better understand the true extent and role of polyploidy in the fishes. However, there are significant challenges to determining the potential advantages of polyploidy in the fishes. In allopolyploidy, it is difficult to separate the effects of polyploidy with those of hybridization. In an established autopolyploid species, differing directions of evolution with that of diploid relatives can obscure the effects of polyploidy. There is a potential for using artificially produced polyploid fish to determine the immediate advantages of polyploidy (Leggatt and Iwama, 2003).

1.2.1. Autopolyploidy and allopolyploidy

Polyploid organisms can originate either from alterations of meiotic or mitotic processes in specimens within a species (autopolyploidy) or by reproductive contact species (allopolyploidy) (Piferrer et al., 2009). Autopolyploidy (mostly autotriploidy and autotetraploidy), occurs from three main mechanisms: a) dearrangements of gametogenesis caused by cytogenetic alterations of meiosis (Cherfas et al., 1995), or nondisjunction of mitotic chromosomes during embryo cleavage; b) suppression of the second meiotic division due to cytoskeletal alterations in postovulatory, aged oocytes (Varkonyi et al., 1998; Aegerter and Jalabert, 2004; Aegerter et al., 2004, 2005; Ezaz et al., 2004; Flajšhans et al., 2007);

Table 1. *Chromosome number and genome size of different Acipenseriform species.*

Species	Common Name	Chromosom number	Reference	DNA content (pg.nucleus^{-1})	Reference
Family Acipenseridae					
Acipenser nudiventris	Fringebarbel sturgeon	118 ± 3	Arefjev, 1983	3.88–4.04	Birstein et al., 1993
		118 ± 2	Vasiliev, 1985		
		118 ± 2	Sokolov and Vasiliev, 1989		
		116 ± 4	Nowruzfashkhami et al., 2006		
Acipenser oxyrinchus desotoi	Gulf Sturgeon	–	–	4.55	Blacklidge and Bidwell, 1993
Acipenser oxyrinchus oxyrinchus	Atlantic sturgeon	99–112	Li et al., 1985	4.38	Hardie and Hebert, 2003
		121 ± 3	Fontana et al., 2008a		
Acipenser ruthenus	Sterlet	116 ± 4	Fontana et al., 1975	3.74	Birstein et al., 1993
		118 ± 2	Vasiliev, 1985	4.1	Zhou et al., 2011
		118 ± 4	Rab, 1986		
		118 ± 2	Birstein and Vasiliev, 1987		
		117.3 ± 0.6	Arefjev, 1989a		
		118 ± 4	Fontana, 1994		
		118 ± 9	Fontana et al., 1995		
		118 ± 4	Rab et al., 1996		
		118 ± 2	Suciu and Ene, 1998		
		118 ± 4	Fontana el al., 1999		
Acipenser sturio	European sea sturgeon	116 ± 4	Fontana and Colombo, 1974	3.2	Mirsky and Ris, 1951
		121 ± 3	Tagliavini et al., 1999	3.6	Fontana, 1976
		122 ± 3	Fontana, 2011		

Acipenser stellatus	Starry sturgeon	118 ± 2	Vasiliev, 1985	3.74	Birstein et al., 1993
		118 ± 2	Birstein and Vasiliev, 1987	4.4	Zhou et al., 2011
		118 ± 1	Nowruz Fashkhami, 1996	4.7	Kafiani et al., 1958
		118 ± 2	Suciu and Ene, 1996		
		114	Nowruz Fashkhami, 1999		
		146 ± 6	Chicca et al, 2002		
Scaphirhynchus platorhynchus	Shovelnose sturgeon	112	Ohno et al., 1969	3.50	Ohno et al., 1969
		–	–	4.73	Blacklidge and Bidwell, 1993
Pseudoscaphirhynchus kaufmanni	Amu Darya sturgeon	–	–	3.46–3.48	Birstein et al., 1993
Huso huso	Beluga	116 ± 4	Fontana and Colombo, 1974	2.42–2.45	Birstein et al., 1993
		109–112	Burtzev et al., 1976	3.6	Fontana, 1976
		118 ± 3	Serebryakova et al., 1983		
		118 ± 2	Birstein and Vasiliev, 1987		
		118.6 ± 0.5	Arefjev, 1989a		
		117.6 ± 0.4	Arefjev and Nikolaev, 1991		
		116 ± 1	Nowruz, 1996		
		120 ± 8	Fontana et al., 1997		
		118 ± 2	Fontana et al., 1998		
		117	Nowruz and Khosroshahi, 1999		
Huso dauricus	Kaluga	120	Serebryakova, 1970	3.74–3.81	Birstein et al., 1993
		270	Vasiliev et al., 2008	8.3	Zhou et al., 2011
		268 ± 4	Vasiliev et al., 2009		
		268 ± 4	Vasiliev et al., 2010		

Acipenser baerii baerii	Siberian sturgeon	249 ± 5	Vasil'yev et al., 1980	8.29–8.31	Birstein et al., 1993
		246 ± 8	Fontana, 1994	8.0	Zhou et al., 2011
		246 ± 10	Fontana et al., 1997		
		229–240	Fopp-Bayat et al., 2006		
Acipenser fulvescens	Lake sturgeon	262 ± 6	Fontana et al., 2004	8.9	Blacklidge and Bidwell, 1993
		–	–	8.0	Zhou et al., 2011
Acipenser gueldenstaedtii	Russian sturgeon	250 ± 8	Vasiliev, 1985	7.86–7.88	Birstein et al., 1993
		250 ± 8	Birstein and Vasiliev, 1987	4.2/8.4	Zhou et al., 2011
		249.9 ± 2.2	Arefjev and Nikolaev, 1991		
		228–268	Arefjev, 1989b		
		256 ± 8	Fontana et al., 1995		
		258 ± 4	Fontana et al., 1996		
		236 ± 2	Hong-bin et al., 2006		
Acipenser medirostris	Green sturgeon	249 ± 8	Van Eenennaam et al., 1999	13.93–14.73	Birstein et al., 1993
		–	–	7.8–8.3	Birstein, 1993
		–	–	8.82	Blacklidge and Bidwell, 1993
Acipenser mikadoi	Sakhalin sturgeon	264	Vasil'ev et al., 2008	8.20	Zhou et al., 2011
		247 ± 33	Vishnyakova et al., 2008		
		262 ± 4	Vasil'ev et al., 2009		
		262 ± 4	Vasil'ev et al., 2010		
		248	Zelenina et al., 2009		
		268	Zhou et al., 2013		
Acipenser naccarii	Adriatic sturgeon	239 ± 7	Fontana and Colombo, 1974	5.7–6.3	Fontana, 1976
		246 ± 8	Fontana, 1994	8.48	Vialli, 1957
		246 ± 8	Fontana et al., 1995		
		241 ± 3	Arlati et al., 1995		
		248 ± 4	Fontana et al., 1999		

Acipenser persicus	Persian sturgeon	> 200	Nowruzfashkhami, 1996		
		258 ± 4	Nowruzfashkhami, 2000		
Acipenser sinensis	Chinese sturgeon	264	Yu et al., 1987		
		156–276	Ye et al., 1999		
		264	Zhou et al., 2008		
Acipenser schrenckii	Amur sturgeon	240	Vasiliev et al., 1980	8.2	Zhou et al., 2011
		238 ± 8	Song et al., 1997		
		266 ± 4	Vasil'ev et al., 2010		
Acipenser transmontanus	White sturgeon	237–243	Hedrick et al., 1991	10.20	Hinegardner, 1976a
		248 ± 8	Fontana, 1994	9.46	Blacklidge and Bidwell, 1993
		226–288	Sola et al., 1994	9.0	Zhou et al., 2011
		246 ± 10	Fontana et al., 1997		
		271 ± 2.5	Van Eenennaam et al., 1998		
		256 ± 6	Wang et al., 2003		
Acipenser brevirostrum	Shortnose sturgeon	254–372	Kim et al., 2005	13.08	Blacklidge and Bidwell, 1993
		372 ± 6	Fontana et al., 2008b	13.78	Hardie and Hebert, 2003
		–	–	13.78	Hardie and Hebert, 2004
Family Polyodontidae					
Polyodon spathula	American paddlefish	120	Dingerkus and Howell, 1976	3.17	Birstein et al., 1993
		120	Symonová et al., 2010	3.90	Tiersch et al., 1989
		–	–	4.89	Blacklidge and Bidwell, 1993
		-	-	3.6	Zhou et al., 2011

Data on sturgeons chromosome number was mainly taken from unife.it/dipartimento/biologia-evoluzione/progetti/geneweb (Fontana, 1994).
Data on sturgeons genome size was mainly taken from genomesize.com (Gregory, 2014).

c) disruption of the process of gamete fertilization by, for example, polyspermy (Kirankumar and Pandian, 2004; Grunina et al., 2006).

Some of these disruptions of the normal meiotic process that lead to polyploidisation have become evolutionarily fixed and made possible the development of polyploid taxa of different levels (species, genera, families; Legatt and Iwama, 2003; Le Comber and Smith, 2004; Comai, 2005; Fontana et al., 2001).

According to reviews of Flajšhans (2006) and Piferrer et al. (2009), origin of allopolyploid fish individuals (mostly allotriploids and/or allotetraploids) is associated to gyno- and/or hybridogenetic mechanisms during interspecific or intergeneric hybridization. Generally, natural interspecific hybridization appeared to be quite common among fish species (Wladytchenskaya and Kedrova, 1982; Benfey, 1989; Vrijenhoek et al., 1989; Bullini, 1994; Scribner et al., 2000). Following hybridization of closely related fish species, embryos and fry often exhibit subtle morphologic differences or they do not show any differences at all. In other cases, e.g. after hybridization of more distant parental fish species with not compatible genomes differing in chromosome number, size and morphology can cause genome reorganizations and modifications. Thus, hybridization can result in genomic alterations, gynogenic and/or androgenic development (Stanley and Jones, 1976) or polyploidization (Chevassus, 1983). Various results in obtaining allotriploid (Arai, 1988; Benfey, 1989; Vrijenhoek et al., 1989; Pandian and Koteeswaran, 1998) and alltetraploid fish (Cherfas et al., 1994; Liu et al., 2001) were reviewed.

1.2.2. Role of polyploidy in evolution

It is noteworthy that there are some difficulties in compiling the known cases of polyploidy in the fishes. Polyploidy is an ongoing process, where ancient events can be obscured in further karyotypic evolution, and more recent events can lead to multiple ploidy levels in one species (Leggatt and Iwama, 2003). Lampreys (Cephalaspidomorphi), shark and rays (Elasmobranchi), and sturgeon (Acipenseriformes) have chromosome numbers much greater than the modal number of 48, which has been postulated to be due to ancient polyploid events (Ohno et al., 1968; Stingo and Rocco, 1991; Birstein et al., 1997). It is generally accepted that polyploidy signifies the presence of three or more ploidy levels. At the same time, the origin of sexual reproduction about one milliard years ago (Smith, 1978) resulted in the first diploid zygotes. This process should be considered the first stage of polyploidization among mega evolution events (Vasil'ev, 2009).

Two diametrically opposing views exist, one assigning polyploidy a marginal role in evolution and the other one granting it a primary creative role. On the one hand, polyploids may represent a relatively frequent class of mutation, one that occasionally establishes within populations when its phenotypic effects are relatively mild. Simply put, polyploidy may be widespread because it arises repeatedly, without playing a significant role in evolution. Conversely, polyploidy may be common because polyploid species evolve faster or in more novel directions than related diploid species (Otto and Whitton, 2000).

Formerly, polyploidy was considered of no importance in vertebrate evolution and also quite impossible among these animals. Muller (1925) considered a developed mechanism of sex determination a substantial barrier for the origin of polyploid species, and he was perhaps the first who advanced arguments for the impossibility of polyploidy among bisexual animals. Later, White (1946) and Mayr (1963) assumed polyploidy in parthenogenetic species, on the basis of numerous data. The development of cytogenetic methods and their wide utilization in taxonomic, phylogenetic, and evolution investigations of vertebrates from the beginning of the 1960s has resulted in the discovery of polyploidy not only in parthenogenetic forms, but

also in bisexual species (fish, amphibians) in which origin of polyploidy is of main significance (Vasil'ev, 2009).

The large amounts of recently accumulated diverse data confirm polyploid evolution in acipenseriform fishes. The problems of ploidy levels, as well as the ways of polyploidization, are unresolved questions related to polyploid evolution in this group (Vasil'ev, 1985). One of the biggest stumbling blocks to the successful establishment of polyploidy in sexual species is the requirement for a genetically compatible mate. Perhaps because of this obstacle, successful polyploid establishment appears to be facilitated by selfing, asexuality, and perenniality (Bell, 1982; Ramsey and Schemske, 1998; Stebbins, 1938). In predominantly outcrossing taxa, an obstacle facing newly formed tetraploids is that they often mate with diploid relatives, producing triploids. Triploidy has generally been thought to be an evolutionary dead-end, because triploids have very low fertility and tend to produce aneuploid gametes, owing to problems of chromosomal pairing and segregation during meiosis. Nevertheless, triploids do produce euploid (haploid, diploid, or triploid) gametes at a low rate (Ramsey and Schemske, 1998; Schultz, 1980). These euploid gametes can then lead to the production of triploid and tetraploid offspring (Otto and Whitton, 2000). This fenomen was also investigated in stocks of sturgeons (*A. ruthenus, A. gueldenstaedtii, A. baerii*), where were found evolutionary tetraploid, hexaploid, pentaploid, heptaploid and octoploid sturgeon fishes. Flajšhans and Vajcova (2000) hypothesized that hexaploid specimens originate from interspecific hybridization of tetra- and octaploid sturgeon species. In this case pentaploid specimens may originate from a backcross of hexaploid hybrid and tetraploid parental species, while heptaploid specimens may have originated from similar backcross of hexaploid hybrid and octaploid parental fish.

An autopolyploid, and especially autotetraploid bisexual vertebrate species may be formed from three main mechanisms of autopolyploidization.

1. A diploid egg may be fertilized by diploid sperm. Such an event has an infinitesimal possibility. For example, spontaneous egg diploidization (usually by the junction of pronuclei in haploid egg as a result of suppressing the second meiotic division) occurs regularly in several fish species, but with low frequencies: according to data it is close to 0% in acipenserids. There are no data on spontaneous diploidization in sperm, but peculiarities of cytological processes of sperm development allow the consideration of its frequency to be close to 0. Moreover, this way of tetraploidization will result in unisexual progeny: all-male with male heterogamety or all-female with female heterogamety.

2. A normal diploid zygote may be subjected to further endoreduplication of chromosomes during the first division. For the production of both male and female progeny, the simultaneous origin of at least two tetraploid specimens of different sexes is necessary. Experimental results demonstrate that autotetraploids are viable in many amphibians and most fishes. At the same time, the crossing of autotetraploid and diploid specimens resulted in triploids in several experiments.

3. The third way of tetraploidization includes two stages. The first stage results in the origin of autotriploid specimens producing triploid eggs. The fertilization of these eggs at the second stage results in the origin of tetraploid specimens. Spontaneously originated autotriploids are periodically found during karyological studies in different fish species (Vasil'ev, 1985). Possibly, they resulted from the fertilization of diploidized egg (see the first way of tetraploidization). To date, well-known technologies to obtain autotriploid progenies (usually by using thermal shock after insemination to depress the second division) have been developed and applied in aquaculture. The sterility of

these progenies increases their meat productivity. However, due to sterility, the origin of autotetraploids by this method seems problematic.

Thus it can be seen that the origin of autopolyploids by the first and the third methods is practically impossible. Only the second method deserves attention, but this method has two important limitations: (1) at least two tetraploid specimens with different sex should originate at the same time; (2) these tetraploid specimens should be isolated from diploids by ecological barriers (for example, by the presence of small isolates). Moreover, the probability of meiotic disturbances with significant and negative influences on fertility is quite high. In any case, this probability is much stronger than in allotetraploids.

According to available data, the origin of polyploid species by hybridization seems the most realistic. There are two main ways of allotetraploidization: direct and indirect, related with clonal heritability. Three main mechanisms are basic for the direct method of allotetraploidization.

1. The fertilization of a diploid egg from one species with a diploid sperm from another species. This, as in the case of autopolyploidy, will result in unisexual progeny, but the probability of this process is infinitesimal.
2. The hybridization between diploid species with subsequent production of unreduced gametes and their further junction. The production of unreduced gametes is the main characteristic of clonal vertebrates that have hybrid origin and are known among fish, amphibians and reptiles. Actually, this characteristic ensures clonal heritability. However, the origin of tetraploid species by this method seems improbable, since only one sex is fertile in interspecific hybridization (Haldane, 1922; Nurelli, 1998).
3. The origin of tetraploid specimens by interspecific hybridization and duplication of chromosome number during the first division, but this seems improbable for the same reasons as the origin of autotetraploids in the same way.

The hypothesis of indirect polyploidization should be considered as the most suitable among the various hypotheses on the origin of bisexual polyploid vertebrate species. It presumes two similar ways. According to the first, bisexual tetraploid vertebrates may result from hybridization between diploid bisexual species and triploid gynogenetic (parthenogenetic) forms of hybrid origin. At present, allotetraploid forms that originated in this way are known among different vertebrates.

The mechanisms of polyploidization (including successive events resulting in ploidy increase) are well known in unisexual vertebrates (Vasil'ev, 1985; Vrijenhoek et al., 1989), but remain unclear in bisexual vertebrates. This is caused by the ancient origin of polyploid bisexual species (usually tens or even hundreds of millions years ago) and the subsequent evolutionary changes in polyploid genomes, as well as the uncertainty of the specific structure of animal groups subjected to polyploidization events. Thus, the greatest step in understanding the origin of bisexual polyploids is the presentation of a hypothesis concerning the type of polyploidy (allo- or autopolyploidization) based on any data on karyotype structure, meiosis, or genetic markers (Vasil'ev, 1985).

Available data on the evolution of polyploid species do not present distinct proof about possible mechanisms of tetraploidization in fish, either allo- or autopolyploidy and a definite way of occurrence. In the case of allopolyploidy, only segmental allopolyploidy (summarizing genomes with partially homologous chromosomes) might have occurred (Vasil'ev, 2009).

1.2.3. Induced polyploidy

During the last three decades, approaches based on interspecific hybridization, sex and ploidy manipulation techniques have been adapted to varying extent by the sectors of commercial aquaculture. In modern finfish aquaculture, high growth rates cause captive animals to reach puberty earlier than their wild conspecifics. As a result of a sexual maturation, problems associated with higher probability of disease outbreak, or changes in organoleptic quality of edible parts of fishes were resolved in conspecific polyploids.

Different types of ploidy induction are possible due to manipulations with pre – embryonic events, such as the process of insemination, fertilization, the second polar body extrusion and the first mitotic cleavage. Similarly, mechanisms leading to spontaneous polyploidization also could be neither explained, nor experimentally simulated without understanding these processes. Therefore, knowledge necessarily prerequisite to understand and carry out polyploidization of fish (Flajšhans, 2006).

Triploidy is artificially induced when freshly inseminated eggs during the second meiotic division are exposed to a shock causing depolymerization of polymers forming microtubuli essential for the formation of the spindle apparatus (Gaillard and Jaylet, 1975, Streisinger et al., 1981) followed by extrusion of the second polar body. As a result, spindle formation is inhibited and the second polar body is retained. Triploids result if the unreduced egg nucleus fuses with the sperm nucleus (Ihssen et al., 1990). Genome of such triploid individuals possess one paternal chromosome set (from the male pronucleus) and two maternal chromosome sets. In principle, shocks to induce triploidy are identical with or similar to those used to restore diploidy during meiotic gynogenesis. Pandian and Koteeswaran (1998) point out notable exceptions from the above scheme in triploids obtained by mating tetraploid males to diploid females (Chourrout et al., 1986; Blanc et al., 1987; Arai et al., 1993; Matsubara et al., 1995) or by dispermic fertilization of haploid eggs (Ueda et al., 1986). In these cases, triploids possess one set of maternal chromosomes and two sets of paternal chromosomes. It is also possible to have vice versa scheme when triploids are obtained by mating of tetraploid females with diploid males.

Tetraploidy is artificially induced in zygote undergoing the first mitosis, i.e. shortly before the first cleavage. In this case, the zygote undergoes genomic replication but the shock inhibits cytokinesis (Pandian and Koteeswaran, 1998). Genome of such tetraploid individuals possess two paternal and two maternal chromosome sets. Shocks to induce tetraploidy are identical with or similar to those used to restore diploidy during mitotic gynogenesis and androgenesis (Flajšhans, 2006).

Sexual maturation usually results in decreased body growth rate since fish divert their energy towards gonadal development. It also often associated with higher incidence of diseases, as in the turbot (*Scophthalmus maximus*), or changes in the organoleptic properties the edible parts, as in many salmonids. These problems can be avoided by producing sterile animals. Sterility may be achieved by the induction of polyploidy, particularly triploidy. Polyploidy is also applied to shellfish species, especially molluscs such as the Pacific oyster (*Crassostrea gigas*), to increase growth rate and/or to improve organoleptic quality (Nell, 2002). Sterility produced by triploidy also has a significant potential applications in the genetic containment of aquaculture species (Piferrer et al., 2009).

Understanding the physiology of polyploids might help to define optimal rearing conditions and to clarify possible causes of their inferior growth, incidence of deformities or lower survival rates following disease outbreaks. Studies on gonad histology, sexual maturation and gamete physiology of the hypothetically sterile fish and of their possible interactions with wild ichthyocoenoses by unwanted hybridization, predation or competition might help to

assess the impacts of escaped farmed polyploids, intentionally stocked triploids for "trophy" fishing or for biological control purposes of transgenic fish (Flajšhans et al., 2006).

Hybridization is more infrequent among marine fish than among freshwater fish (Vasil'ev, 2009). Besides, freshwater differs from marine environments in the probability of creating small isolates, for example during seasonal water-level changes. Such freshwater isolates represent strong factors for interspecific hybridization and further fixation of evolutionary events.

1.3. GENOME SIZE AND CELL SIZE

Studies indicate that perhaps the simplest demonstration of the relationship between DNA content and cell size is that provided by polyploidy. In this case, entire chromosome sets are duplicated such that differences in DNA content among species are large and easily quantified. Polyploidy has long been known to result in large number of brain, retina, epithelium, cartilage, muscle, liver, kidney, testis, ovary and blood cells (Fankhauser, 1955; Licht and Bogart, 1987; Benfey, 1999). Today erythrocyte size is often used as a means of identifying polyploid individuals (Austin and Bogart, 1982; Garcia-Abiado et al., 1999). The relationship between genome size and erythrocyte size in fish is so far the least well established and needs further investigation.

Lay and Baldwin (1999) present data for nuclear and cell volumes for 52 tropical species of teleost fish and conclude that no significant relationship exists between these two parameters. This purported counterexample may simply reflect problems with the chosen methodology. Specifically, Lay and Baldwin (1999) measured wet cell volumes by dividing hematocrit by cell counts, and compared this against dry nuclear "volumes" calculated from two-dimensional measurements of Feulgen-stained nuclei. The error inherent in this comparison aside, these authors assumed a three-dimensional ellipsoid shape of nuclei when calculating nuclear volumes from length and width measurements – clearly an unjustified assumption given the profound flattening that occurs when blood smears are dried. Unfortunately, genome sizes are not known for the species studied by Lay and Baldwin (1999), so a more direct comparison of the relationship between cell size and DNA content is not yet possible for this group (Gregory, 2001a).

There are many ways in which erythrocyte (red blood cell, RBC) size is of relevance to organismal biology. Larger RBCs contain more hemoglobin (Hawkey, el al., 1991), but they also require larger blood vessels. Species with large cells also typically have fewer cells (Hawkey, el al., 1991; Kuramoto, 1981). Blood viscosity, total hemoglobin content, and other such parameters are of obvious significance to organismal physiology, but no other parameter has received more attention in regards to genome size/cell size interactions than erythrocyte surface area to volume (SA:V) ratios (Gregory, 2001a).

The significance of erythrocyte SA:V ratios to organismal metabolism was discussed in some detail by Harvey Smith in 1925: "The fact that the mass of body increases as the cube of the linear dimension, while the surface increases as the square, has long been recognized as of importance in biology. It is only a step farther to apply the same idea to cell size." Since then, the notion of erythrocyte size directly influencing organismal physiology has been applied unilaterally among the vertebrate classes. Smith (1925) discussed the association between cell size and metabolic rate in amphibians, and the large cells and genomes of aquatic urodeles and lungfishes have long been interpreted as adaptations for life in hypoxic environments (particularly as related to aestivation) (Cavalier-Smith, 1991; Gregory and Hebert, 1999).

Instead, in fish it appears that genome size is associated with some measure of "developmental complexity." That is, small genomes are typical of complex species which

deviate significantly in their morphology from the typical "fishy" design (Hinegardner 1968, 1976b). One plausible explanation for this is that the number of steps to be carried out in a complex developmental program like that of a seahorse exceed those of a "fishy" fish like a trout, and that cell division and differentiation must therefore be faster (and genomes smaller) in the former. Thus, although a relationship clearly exists between genome size and erythrocyte size in fish, this may be more of a secondary consequence than a primary target of selection in this class (Gregory, 2001a).

The various explanations for the correlation between genome size and cell size each fall into one of three broad categories: mutation pressure theories, the nucleoskeletal theory, or nucleotypic theory.

1.3.1. Mutation pressure theory

In the first of these, genome sizes are viewed as the product of an ongoing process of DNA accumulation (i.e., an upward mutation pressure) halted only when the replicative costs exceed the tolerance of the "host" cell. Under these theories, it is generally assumed that larger cells can simply tolerate more DNA, thereby making this correlation merely coincidental. The two main examples of this approach include the selfish DNA theory and the junk DNA theory, both of which are of substantial utility in addressing the origin and spread of non-coding DNA, but which falter with regard to the cell size relationship. In the simplest terms, there is no reason to expect large cells to be proportionately more tolerant of non-coding DNA than smaller cells (Gregory, 2001b). As an alternative approach, optimal DNA theories postulate some physiological, cytological, or other such role for non-coding DNA. Possible function (or at least, an effect) of bulk DNA that is independent of its specific nucleotide sequence. In particular, optimal DNA-based explanations for the correlation between genome size and cell size typically center on the relationship between these two parameters and nucleus size. This relationship, for its part, has been known since the last turn of the century as either the "cytonuclear ratio" or the "karyoplasmic ratio" (Wilson, 1925 and earlier studies).

1.3.2. The nucleoskeletal theory

Under the "nucleoskeletal theory," cell size is not determined by nucleus size, but rather is set adaptively by genes. The correlation between nucleus (and therefore genome) size and cell size arises through a process of coevolution in which nuclear size is adjusted to match alterations in cell size (Cavalier-Smith, 1982, 1985). The nucleoskeletal theory outlining the mechanism by which DNA content can causally influence nucleus size (Cavalier-Smith, 1982; Gregory 2005). Thus, DNA acts as a "nucleoskeleton" around which the nucleus is assembled, with the total amount (including both coding and noncoding sequences) and compaction level exerting a strong effect on the final nucleus size. Such a view is supported by trans-specific DNA injection experiments, which show nucleus-like structures to form around DNA, regardless of its source (e.g., Forbes et al., 1983).

The overall output is to be increased the number of each must be increased. At the moment the nucleoskeletal theory emphasis on the needs of larger cells to meet the higher demands for proteins, but there is no reason to expect this to scale in direct proportion to cell size (Beaton and Cavalier-Smith, 1999; Cavalier-Smith and Beaton, 1999). Even in cases where nucleus/genome size and cell size are matched proportionately, there is nevertheless a negative relationship between genome size and cell growth/division rate. Within this theory, it was proposed that cell size variation always be considered adaptive, which is a very difficult assumption to uphold in several cases. Interpretation of the cell size correlation, seems

rather problematic and has not been widely accepted. Instead, most theorists have tended to extend the idea of causation to the cell level as well, usually via the intermediate of nucleus size (Gregory 2005).

1.3.3. The nucleotypic theory

Term "nucleotype" was coined by Bennett (1971) to describe "that condition of the nucleus (DNA content) that affects the phenotype independently of the informational content of the DNA." Since then, the "nucleotypic theory," which is also an optimal DNA theory, has become the most widely implemented approach to understanding the relationship between genome size and cellular/organismal features. On a certain level, DNA content and cell size must be causally related, due to the physical impossibility of containing very large genomes within small cells. Although direct evidence in favor of the theory is not yet available, but it was supported by the fact that polyploidization results in an instantaneous and proportionate change in cell size, and diploid laboratory hybrids tend to show intermediate genome and cell sizes relative to their parental species, again suggesting a causative link between the two parameters. One of the most recent explonations have been proposed by Nurse (1985). It was poining out that "cell size is determined by an interaction of the function of specific genes with the total DNA content of the cell," and that "such an interactive system can be best understood in terms of cell cycle controls which coordinate progress through the cell cycle with an increase in mass." Under the recent "gene-nucleus interaction model", bulk DNA influences the space-filling requirements for cyclins in larger nuclei, and possibly even the influx of regulatory proteins (and therefore cyclin gene expression) owing to effects on nuclear surface area to volume ratios and/or the arrangement of chromatin within the nucleus (Gregory, 2001a). Importantly, and unlike the coincidental and coevolutionary approaches, such a nucleotypic model is applicable to all cell types, including those in which the nuclei are ejected during final differentiation (Gregory 2005).

A general relationship between DNA content (or at least, nucleus size) and cell size has been known for over a century. However, the explanation for this association remains a subject of debate even today.

1.4. MEASURING FISH PLOIDY LEVEL

The ploidy level was commonly estimated using 'direct' methods that are based on determining amount of DNA in cell nucleus. Most common of these include karyotyping, complete genome sequencing, quantification of relative or absolute DNA content in stained cell nuclei by means of flow cytometry (FC), Feulgen image analysis densitometry (FIA) and/or microdensitomery. Another possibility for ploidy level estimation is based on checking the size of cells and/or their nuclei and known as 'indirect'. These include measuring cell/nuclear geometry using light, fluorescence or confocal laser scanning microscopy (CLSM), Coulter counter analysis or by classic hematology. Majority of frequently used methods for ploidy level, genome size and morphology definition reviewed in details in following text.

1.4.1. Karyotype

Karyotyping is a technique that allows geneticists to visualize chromosomes using proper extraction and staining techniques when the chromosomes are in the metaphase portion of the cell cycle (Fergus, 2009). Karyotype analysis can be performed on virtually any population of rapidly dividing cells either grown in tissue culture or extracted from tumors. Chromosomes can be prepared in invasive or non-invasive way. From sacrificed fish it is possible to use cranial part of kidney, spleen, gonads or gill epithelium. Embryonic and larval tissues can be also used for preparation of mitotic chromosomes (Phillips and Hartley, 1984; Inokuchi et al., 1994). Non-invasive approach allows chromosome preparation based upon blood- and fin sampling, cultivation of lymphocytes (Sanchez et al., 1990; Fujiwara et al., 2001) and fibroblasts (Alvarez et al., 1991). Chromosomes derived from peripheral blood lymphocytes are ideal because they can be analyzed three days after they are cultured. The harvested cells are treated briefly with a hypotonic solution. This causes the nuclei to swell making it easier for technicians to identify each chromosome. The cells are fixed, dropped on a microscope slide, dried, and stained. Chromosome spreads can be photographed, cut out, and assigned into the appropriate chromosome number or they can be digitally imaged using a computer. There are seven groups (A-G) that autosomal chromosomes are divided into based on size and position of the centromere. The total number of chromosomes is written followed by a comma, then the sex chromosome constitution and any abnormality written in parentheses. Karyotypes also may help determine the cause of infertility in patients having reproductive difficulties. The karyotype test is quite complex that's why it takes around a week to get karyotype results. It is strictly specific to each organism and characterized by high degree of constancy and as serves important taxonomical sign as the tool of karyosystematics (Flajšhans, 2006). It can be used for many purposes; such as, to study chromosomal aberrations, cellular function, taxonomic relationships, and to gather information about past evolutionary events.

Fish karyotypes are generally characterized by large numbers of small chromosomes. This discourages many researchers from pursuing fish karyotypic analysis, and therefore, karyological data on fish are available for only a small percentage (about 10%) of some 25,000 species taxonomically known so far (Klinkhardt et al., 1995; Froese and Pauly, 2006). Opening of triploid and tetraploid fish forms allowed to reassess role of hybridization and polyploidy in evolution of vertebrates. Without involving karyological data it would be very difficult or even impossible to address issues of fruitful and phylogeny for many groups of fish (Acipenseridae, Salmonidae, Cobitidae, Cyprinodontidae and others).

Karyotyping is considered to be one of the most accurate method to determine ploidy level (Linhart et al., 2001; Flajšhans, 2006). However, it is also considered to be time demanding, expensive and requiring specific cell culture conditions. Karyotiping may reveal some minor abnormalities affecting only a few chromosomes such as aneuploidy or heteromorphisms in some triple sets, undetectable using other approaches (Flajšhans, 2006).

1.4.2. Flow cytometry

The flow cytometer measures the physical or chemical characteristics of individual cells as they move in fluid stream pass optical or electronic sensors (Melamed et al., 1990). The technique provides a rapid and precise means for detecting, counting and characterizing cells in mixed population (Morgan et al., 1993). Developed in the late 1970's primarily as a means of detecting the anomalous DNA contents of cancer cells, flow cytometry has since become a staple of genome size research. Now flow cytometry allows precise determination of relative and/or absolute DNA content per cell. This technique is based upon staining the cell nuclei

with specific DNA dyes (e.g., propidium iodide or DAPI) capable of absorbing the illuminating light and upon analysis of fluorescence emitted by the dye/DNA complex. Flow cytometry enables to determine ploidy level in native cells of fish embryos (Lecommandeur et al., 1994), larvae (Ewing et al., 1991) and in native blood or tissue cells of older fish specimens (Allen, 1983). This accomplished by passing the stained nuclei through the path of a laser of a specific wavelength, which stimulates the emission of light by the fluorochrome. The fluorochromes used in flow cytometry are base pair-specific, such that differences in GC/AT content can affect the measurements if only one stain is used (Vinogradov, 1994, 1998). The results of analysis are usually displayed in the form of a histogram or dotplot of fluorescence intensity among the nuclei in the sample. The technique has important limitations related to the large number of nuclei required for analysis and the need to place them in suspension. When blood cells are used, very large cell populations can be sampled from a single individual. However, when small organisms are to be studied, the large cell numbers required for flow cytometry can present a problem (Korpelainen et al., 1997). In many cases specimens are either not available in these numbers or the issue under investigation requires genome size estimates for single individuals, or even tissue-specific estimates.

Apart from native cells used for ploidy level assessment, fixation technique (Gold et al., 1990) as cryopreservation of isolated blood cells (Fisher et al., 1995) was considered as a good blood preservation method for future flow cytometry measurements. Ethanol fixation of the blood sturgeon cells was found suitable but it can result in production of higher variation coefficients and background noise signal (Birstein et al., 1993). Repeated measurements of samples are not possible because the procedure does not produce permanent preparations and fluorescence fades quickly. Flow cytometry is also typically limited to the inclusion of only one standard, and problems with staining can therefore be difficult to detect.

Nowadays flow cytometry became broadly applied for rapid and cheap ploidy level determination in studies of various polyploid hybrid species (Ráb et al., 2000). Flow cytometry is currently the most efficient and accurate method available for genome quantification, it combines advantages of densitometric technics as permanent and easily prepared specimens, cissue-specific measurements, multiple standards, low cost. However, the cost of equipment is a major challenge his ubiquitous.

1.4.3. Feulgen image analysis densitometry

The histochemical reaction developed by Robert Feulgen was used for the detection of DNA in the nucleus (Feulgen and Rossenbeck, 1924), but since the demonstration that it is both specific and stoichiometric for DNA it has become the most important means of staining nuclear DNA for densitometric quantification. The protocol has been modified frequently and substantially, but the basic components have not been altered. The most commonly employed stain in the Feulgen reaction is Schiff's reagent, developed by Hugo Schiff in the 1860's (Schiff 1866). Schiff reagent has been prepared in a variety of ways; most modern preparations follow a modified version of the protocol developed by Lillie (1951), which uses sodium (or potassium) metabisulfite as a decolorizing agent. Schiff reagent is available commercially, but it is not as reliable as freshly prepared solutions. Almost every step in the Feulgen reaction procedure has been varied among studies, including sensitivity to the choice of fixative and fixation time, concentration and temperature of acid and hydrolysis time. Each of these can affect the efficacy of staining (Kasten, 1960; Deitch et al., 1968; Kjellstrand, 1980; Schulte, 1991).

Feulgen densitometry relies on the simple premise that the amount of stain bound is directly proportional to the amount of DNA present. The quantity of stain is itself determined based

on the amount of light it absorbs (i.e., its density). It is not possible to measure absorbance directly. Instead, absorbance (optical density) must be calculated indirectly from measurements of the amount of light passing through the object (transmittance). Transmittance, in turn, is measured as the difference between the intensity of incident light entering the object and that of the transmitted light leaving it that is why measurements are taken both within the nucleus and outside the nucleus in a clear area of the slide. The heterogeneous nature of DNA stain in the nucleus means that any single point measurement will not be representative of the nucleus as a whole that is why it is necessary to take a series of point densities covering the entire nuclear area. The sum of these individual optical densities is the integrated optical density (IOD). Image analysis software uses individual pixel values to instantaneously calculate IOD from the image as a whole. This approach not only avoids the necessity of acquiring individual point densities one at a time, but it also allows the simultaneous tabulation of IODs for all of the nuclei within a microscope field (Hardie et al., 2002).

1.4.4. Molecular genetic techniques

Enzyme electrophoresis is potentially powerful in resolving genetic proximity, employing the degree of polymorphism of diverse alleles at different loci involved in translating specific enzymes and their varied multiple molecular forms. These were used as molecular tags in studies on the identification of polyploidy type and strains (Liu et al., 1978; Arai, 1988; Pandian and Koteeswaran, 1998 and others). This technique is based upon isolation of proteins from respective tissues, their separation by gel electrophoresis and staining for one or more allozymes/isozymesby. For better understanding, Beaumont and Hoare (2003) exemplified this approach on a triploid heterozygote bearing two alleles (AAB), which could be distinguished from a diploid (AB) by stronger staining of the band produced by the double allele. They pointed out that use of microsatellite loci meant a great improvement on allozymes due to higher heterozygosity, as they were more likely to show three bands.

Han et al. (1992) used DNA fingerprinting for identification of ploidy in ayu, *Plecoglossus altivelis*. The importance of DNA fingerprinting as a tool in fish biology was further pointed out e.g. in the review of Pandian and Koteeswaran (1998). In a recent study, Sousa-Santos et al. (2005) used DNA sequencing and relative sizes of double peaks in chromatograms to determine ploidy level and relative contribution of the parental genomes in hybrids (Flajšhans, 2006).

Although thousands of DNA amounts have been determined using the previously discussed techniques over the past 50 years, every one of them is but an estimate, inevitably subject to technical errors. For this reason, the need for an exact calibration standard who's C-value is not subject to such errors has long been recognized. Since the mid-1990s, a large number of highly accurate determinations of genome size based on complete genome sequences have been published. Because of the high cost and intensive effort currently required, it is unlikely that complete genome sequencing will become a viable and routine method for determining genome size in the near future (Gregory, 2005).

1.4.5. Erythrocytes' geometry

Erythrocyte geometry parameters were under study during last couple of centuries in variety of plants and animals. These parameters usually include studies of simple forms as points, lines, surfaces, solids, and higher dimensional analogues. Biggest interests usually belong to studies that were able to show and describe relationships between geometry and different biological characteristics as respiration, DNA concentration, chromosome packaging

and many others. Nowadays there is number of rooted methods to study fish erythrocytes geometry. One of the most common methods is based on light or fluorescence microscopy measuring erythrocyte dimensions from dried stained blood smears or from stained live cells. Both microscopic technics showed that the size discrepancies between live and fixed erythrocytes had very little individual effect on resulting morphometric variables. Similar results are obtained for longitudinal and transverse axis that were also reported as major and minor axis showing that fish erythrocytes and their nuclei made from blood smears reflect the true dimensions of live cells. It was also shown that erythrocyte nuclear area and perimeter were the most precise ploidy level predictors (Flajšhans et al., 2011). Volume of particles is usually calculated using major and minor axis which is also used as third axis. In addition, difference in dimensions of erythrocytes in fishes with different ploidy level allows identifying ploidy level in number of fish species. These relationships were first shown by Swarup (1959) to recognize diplod and triplod fishes. Relationships between diploids and triploids fishes were shown by number of authors (Krasznai et al., 1984; Ueno, 1984; Benfey et al., 1984; Cherfas et al., 1991), the same idea was used to identify ploidy level in polyploid sturgeons (Austin and Bogart, 1982; Wolters et al., 1982; Benfey et al., 1984; Garcia-Abiado et al., 1999). Another method to study erythrocytes volume and other geometrical characteristics is based on confocal laser scanning microscopy (CLSM), allowing creating volumetric images of live erythrocytes or their nucleus. Main CLSM principle is similar to tomography. The key feature is ability to acquire in-focus images from selected depths, a process known as optical sectioning. After the scanning process images can be processed in software allowing calculating volume using voxels (Abramoff, et al., 2004; Bolte and Cordelieres, 2009), DNA content using fluorescence spectrum at in each point of our sample and other geometrical and non-geometrical characteristics. Advantage of CLSM, in comparison with other methods; lie in possibility getting more detailed information concerning volume, shape, surface and visualization of erythrocyte or erythrocyte nuclei. This information can give an indication on changes in nucleus or cell structure with increase in ploidy level and other parameters. Visualization of erythrocyte 3D dimension can be also done by mean of electronic microscopy, but this method is more time consuming and cannot be run in regular bases for big number of measurements.

One more possibility to investigate erythrocyte nuclear volume distribution is Coulter counter principle that was invented by Coulter brothers in 1950's (Graham, 2003). According to this principle particles are caused to move through a small constricted electric current path in the suspending fluid and detection is based upon difference in electrical conductivity between the cell and suspending fluid. When a particle is pulled through the orifice, the change in resistance is amplified and accumulated by the Coulter Counter. This change in resistance is proportional to the volume of electrolyte displaced and hence to particle size (Coulter, 1956). Nowadays several types of veterinarian hematological analyzers allow counting and measuring various blood cells of fish, amphibians, reptiles or birds using Coulter counter principle. This method was firstly proposed in fisheries for estimation of erythrocyte nuclear volume distribution by means of Coulter Counter Channelyzer. It was used for rapid identification of triploid grass carp to be certified prior to stocking into open waters for weed control purposes (Wattendorf, 1986). Estimation of ploidy level comes out of medium corpuscular volume (MCV) of blood cells which differs between diploids and triploids (Svobodova et al., 1998).

1.5. AIMS OF THE THESIS

1) Study DNA content specificity in fish, using a complete series of polyploid models of fish species and/or their hybrids, from diploidy to tetradekaploidy.

2) To investigate and acknowledge the theoretical expectations of nucleotypic effect of genome size on cell/nuclear size in 2-D and 3-D space, using techniques of flow cytometry, Feulgen image analysis densitometry and confocal laser scanning microscopy.

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CHAPTER 2

USE OF DIPLOID AND TRIPLOID TENCH (*TINCA TINCA*) BLOOD AS STANDARDS FOR GENOME SIZE MEASUREMENTS

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Use of diploid and triploid tench (*Tinca tinca*) blood as standards for genome size measurements

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Summary

The ploidy level of 10 diploid and 10 induced triploid tench, *Tinca tinca,* was verified using flow cytometry to determine relative DNA content of DAPI-stained erythrocyte nuclei. The C-value (haploid nuclear DNA content; pgDNA nucleus^{-1}) of these same individuals was determined by means of Feulgen image analysis densitometry, in comparison to the chicken (*Gallus gallus domesticus*; 1.25 pg $P < 0.05$) standard, using three different approaches. Highly similar mean C-values were obtained, thus confirming the possibility of using tench blood as a frequently used standard in European pond aquaculture for ploidy determination in fishes.

Introduction

Interest in genome size differences among fishes has increased over the past few years (Gregory and Witt, 2008; Ardila-Garcia and Gregory, 2009; Smith and Gregory, 2009; Zhou et al., 2011). In this regard, methods such as flow cytometry and Feulgen image analysis densitometry have become more popular for identification of ploidy level and for measurements of genome size and cell/nuclear sizes (Lu et al., 2009; Flajšhans et al., 2011; Bytyutskyy et al., 2012). For polyploids, the latter term is preferably expressed as the C-value (haploid nuclear DNA content), as they may contain more genomes per haploid nucleus (Smith and Gregory, 2009). Genome size refers to the amount of DNA in one copy of a genome, which is the same as the C-value in diploid organisms (Swift, 1950).

The choice of a standard for such measurements is crucial for the calculation of absolute genome size following Feulgen image analysis. It is desirable to choose standards of the same cell type for the unknowns, although this convention is often ignored. Hardie et al. (2002) showed that the difference in the characteristics of standard vs unknown cells can represent a substantial source of error. For these reasons the standards should be of commonly used species (e.g. chicken, *Gallus gallus domesticus*) or species that have been previously measured using a reliable method such as flow cytometry. Nowadays chicken appears to be one of the most common standards for ploidy level identification (Greilhuber et al., 2007; Mendonca et al., 2010; Bytyutskyy et al., 2012), but because of limited access to the blood of these animals on fish farms it would be convenient to use a fish standard. The purpose of this study was to measure genome size for diploid and induced triploid tench using two staining methods and evaluated under two hardware/software systems, and thereby verify the possibility of using diploid and triploid tench (*Tinca tinca*) as the frequently used standard in European pond aquaculture (Flajšhans and Piačková, 2006).

Materials and methods

Experimental fish

All fish (10 diploids and 10 induced triploids) originated from broodstock of the University of South Bohemia České Budějovice, Faculty of Fisheries and Protection of Waters at Vodňany, Czech Republic. Triploids were randomly sampled from a pilot-scale production (Flajšhans et al., 2010) based upon factorial mating of 50 females and 25 males followed by cold shock according to the protocol of Flajšhans et al. (2004). Diploids were randomly sampled from the control (untreated) group. Prior to any handling, fish were anaesthetized with 0.6 ml l^{-1} 2–phenoxyethanol (Merck Co., Darmstadt, Germany). Blood was collected from the caudal vessel into a heparinized syringe according to Pravda and Svobodová (2003), kept at 4°C and processed immediately after transfer to the laboratory. Six blood smears were made of each specimen, following Pravda and Svobodová (2003).

Standard

Ten samples of chicken blood from 10 different individuals, for use as an external standard, were obtained by courtesy of a poultry processing plant in Vodňany, Czech Republic. All blood smear samples were obtained within a 1-month period.

Flow cytometry

Prior to staining and cell imaging for densitometry, the ploidy level of each fish was first verified as relative DNA content in blood cells by means of flow cytometry (Partec CCA I; Partec GmbH, Münster, Germany) using 4′,6-diamidino-2-phenylindole (DAPI) according to Linhart et al. (2006).

Feulgen image analysis densitometry

All samples were divided into three aliquots and checked by means of the following approaches:

1 The first aliquot was transported to the laboratory of Dr. T. Ryan Gregory (Department of Integrative Biology, University of Guelph, Ontario, Canada) for staining and Feulgen image analysis densitometry following Hardie et al. (2002), using a 3CCD camera Optronics DEI 750 CE coupled to a Leica DM LS compound microscope (objective 100×), and the BIOQUANT TRUE COLOR WINDOWS 98 v.3.50.6 IMAGE analysis software package (R&M Biometrics; Nashville, TN) to measure integrated optical density (IOD) in erythrocyte nuclei.
2 The stained samples were transported back to the authors' laboratory and measured for IOD again using 3CCD Sony DXC-9100P camera coupled to an Olympus BX50 microscope (objective 100×), and the OLYMPUS MICROIMAGE v. 4.0 image analysis software package (Olympus Corp., Tokyo, Japan). Hardware and software setup followed the protocol of Hardie et al. (2002). The software working in the RGB colour model was set only to the green channel with maximal pixel intensity 190; the light intensity calibration was set to Standard Optical Density.
3 The second aliquot of standards was stained using a DNA Staining Kit according to Feulgen (Merck Co.,) and measured for IOD with the latter system.

The IOD was measured in fifty nuclei from each specimen under study and the C-value (pgDNA nucleus^{-1}) was computed according to Hardie et al. (2002).

The effects of fish ploidy on relative DNA content and on the genome size, as well as the measurement precision of both densitometric systems [1 and 2] and of both stainings [2 and 3] were assessed using ANOVA followed by the Tukey's Multiple Comparison Test with the use of STATGRAPHICS v. 5. software. Data were presented as a mean value ± standard deviation (SD). The level of significance was set at $P < 0.05$.

Results

Flow cytometry

The relative DNA content of diploid tench erythrocytes was 50.57 ± 1.71, with mean coefficient of variation (CV) 2.32 ± 0.32%. Triploid DNA content was 1.5-fold higher (75.87 ± 1.57; $P < 0.05$), with CV 2.31 ± 0.54%.

Feulgen image analysis densitometry

Data on the mean C-value of the respective standards and samples are given in Table 1. Briefly, all three approaches to measure the internal standards resulted in highly similar mean C-values of diploid tench (1.00, 1.02 and 1.02 pgDNA nucleus^{-1}, respectively) and in identical mean C-values of induced triploid tench (1.55 pgDNA nucleus^{-1} using all approaches), all at $P < 0.05$.

Table 1
Haploid C-value of Feulgen-stained erythrocyte nuclei of diploid (2n) and triploid (3n) tench, *Tinca tinca*, compared to chicken, *Gallus gallus domesticus*. Data comprise (i) slides stained and measured at University of Guelph, Canada (UoG); (ii) slides stained at UoG and measured in authors' laboratory and (iii) slides stained and measured in authors' laboratory. Mean ± SD; data with same alphabetic superscript do not exhibit significant difference at $P < 0.05$

Approach	Species (ploidy level)	C-value (pgDNA nucleus^{-1})
	Gallus gallus domesticus standard	1.25 ± 0.01
(i)	*Tinca tinca* (2n)	1.00 ± 0.06[a]
	Tinca tinca (3n)	1.55 ± 0.09[b]
(ii)	*Tinca tinca* (2n)	1.02 ± 0.05[a]
	Tinca tinca (3n)	1.55 ± 0.07[b]
(iii)	*Tinca tinca* (2n)	1.02 ± 0.05[a]
	Tinca tinca (3n)	1.55 ± 0.07[b]

Discussion

Both diploid and triploid tench were chosen as internal fish standards because of the wide use of this species as a model fish in the authors' laboratory (Flajšhans et al., 2010). The relative DNA content was found to be in accordance with previous data published by Flajšhans et al. (2004), Linhart et al. (2006) and Bytyutskyy et al. (2012).

The internal standards were used to test the three approaches comparing both systems for Feulgen image analysis densitometry and both sets for DNA staining according to Feulgen. The test revealed high similarity and/or identity of C-values of the diploid and/or triploid tench. It may be concluded that tench can be used as a standard in European aquaculture for identifying ploidy level in fish.

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CHAPTER 3

USE OF FEULGEN IMAGE ANALYSIS DENSITOMETRY TO STUDY THE EFFECT OF GENOME SIZE ON NUCLEAR SIZE IN POLYPLOID STURGEONS

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Use of Feulgen image analysis densitometry to study the effect of genome size on nuclear size in polyploid sturgeons

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Summary

Feulgen image analysis densitometry (FIA) and image cytometry were used to study the relationship between the DNA content (pgDNA nucleus^{-1}) and nuclear area (μm^2) in blood smears of evolutionary tetraploid (4n) sterlet (*Acipenser ruthenus*) and stellate sturgeon (*A. stellatus*); evolutionary octaploid (8n) Siberian sturgeon (*A. baerii*) and Russian sturgeon (*A. gueldenstaedtii*); hexaploid (6n) and decaploid (10n) fish found within *A. baerii* stock; and *A. baerii* and *A. gueldenstaedtii* exhibiting dodecaploidy (12n). Standards used for FIA were blood smears of chicken (*Gallus gallus domesticus;* 2.5 pgDNA nucleus^{-1}) and diploid and induced triploid tench, *Tinca tinca* (2.04 and 3.1 pgDNA nucleus^{-1}, respectively). All ploidy levels were first verified by means of flow cytometry. Species of the same ploidy level, however differing in their DNA content, exhibited a similar mean erythrocyte nuclear area, as could be demonstrated on *A. ruthenus* and *A. stellatus* (19.27 and 19.79 μm^2, respectively) with a respective mean DNA content of 3.72 and 4.68 pgDNA nucleus^{-1} and the same relationship was found for evolutionary octaploid (8n) *A. baerii* and *A. gueldenstaedtii* (29.87 and 30.09 μm^2, respectively) with respective mean DNA content 8.29 and 7.87 pgDNA nucleus^{-1}. The 0.19–0.32 pgDNA increments in DNA content of erythrocytes thus had no effect on their nuclear area. With increasing ploidy level, the DNA concentration (pgDNA per μm^2 of erythrocyte nuclear area) was found not to increase linearly. The DNA in erythrocyte nuclei appeared to be more and more densely packed with an increase of the ploidy level ($r = 0.98$; $R^2 = 0.95$).

Introduction

Research on sturgeon biology is constantly increasing due to their special position in the taxonomy of aquatic organisms, adaptive capacity, high organoleptic characteristics and nutritional value, as well as for the need of their natural reproduction. The genetics are very interesting due to the evolutionary age, fertility, and also for the valuable data on mechanisms underlying the evolution of vertebrates (Fontana et al., 2001).

The past few years have seen a resurgence of interest in the impacts of genome size differences among fishes. In this regard, correlations have been examined recently between genome size and chromosome number (Mank and Avise, 2006), erythrocyte size (Gregory, 2001a,b; Hardie and Hebert, 2003) and others.

According to Gregory (2005), the term 'genome' can accurately be taken to mean the total DNA amount per haploid chromosome set. For diploid organisms, the term 'genome size' refers to the amount of DNA in one copy of a genome, and the 'C-value' (Swift, 1950) simply refers to haploid nuclear DNA content, which is the same thing. But as polyploids may contain two or more genomes per haploid nucleus, the term 'C-value' is used in many cases rather than 'genome size' (Smith and Gregory, 2009). Because the precise meaning of the word 'haploid' is one set of homologous chromosomes (Arefjev and Lisovenko, 1995), it may lead to a misunderstanding when the term 'C-value' is used in the text. Therefore the DNA content, which refers to total amount of DNA per somatic cell, will be used hereafter rather than 'C-value'. In sturgeons as in other fishes, DNA content correlates with a variety of cellular and organismal parameters. Most notable among these is the relationship between DNA content and erythrocyte size. It has been shown that the studies of DNA content variation are important from a number of practical and theoretical perspectives (Hardie et al., 2002). The erythrocyte size is often now used as a means of identifying polyploid individuals (e.g. Austin and Bogart, 1982; Garcia-Abiado et al., 1999).

Shifts in haploid genome sizes are usually much more subtle than changes in ploidy levels, but there is nevertheless a great deal of variation among taxa (Gregory, 2001b). According to this author, as well as to Smith and Gregory (2009), the relationship between DNA content and erythrocyte size is so far the least established in fishes. More detailed analyses and modern equipment can provide sufficient results on genome quantity, polyploidy levels and nuclei and cell sizes of erythrocytes (Piferrer et al., 2009).

Species of genera *Acipenser, Huso, Scaphirynchus* and *Polyodon* are separable into the different classes of chromosome numbers: (i) species with ~ 120 chromosomes, including all taxa with between 110 and 130 chromosomes and 3.2–4.6 pg DNA content in somatic cells; (ii) species with ~ 250 chromosomes, including all taxa with between 220 and 276 chromosomes and 6.1–9.6 pg DNA content of somatic cells; (iii) is represented only by *A. brevirostrum* having ~360 chromosomes and more than 13.1 pg of DNA content in somatic cells (Blacklidge and Bidwell, 1993; Ludwig et al., 2001; Fontana et al., 2008; Vasil'ev, 2009; Havelka et al., 2011; Zhou et al., 2011). These three groups were divided into evolutionary tetraploid, octaploid and dodecaploid-derived groups by Blacklidge and Bidwell (1993) and later confirmed by Birstein et al. (1993), but due to functional genome reduction events, some authors (e.g. Fontana, 1994; Fontana et al., 1998; Tagliavini et al., 1999; Jenneckens et al., 2000; Ludwig et al., 2001) consider all sturgeons with ~120

chromosomes as functional diploid species, species with ~250 chromosomes as functional tetraploid species, and with ~360 chromosomes as functional octaploids. Recently, Fontana et al. (2008) concluded that evolutionary tetraploid, octaploid and dodecaploid species were regarded as functional diploid, tetraploid and hexaploid species, respectively, based on molecular cytogenetic results using fluorescence in situ hybridization (FISH) with different DNA probes.

The purpose of this study was to measure the genome size in sturgeons and sturgeon hybrids of both known and unusual ploidy levels and to estimate the relationships between genome size and selected cell nuclear dimensions for species of the same – as well as of differing – ploidy levels.

Materials and methods

Fish specimens

Altogether the 110 sturgeon samples originated from broodstock of the University of South Bohemia České Budějovice, Faculty of Fisheries and Protection of Waters at Vodňany, Czech Republic. Samples comprised sterlet, *Acipenser ruthenus*; stellate sturgeon, *A. stellatus*; Siberian sturgeon, *A. baerii;* and Russian sturgeon, *A. gueldenstaedtii,* as well as specimens with unusual ploidy levels found within Siberian and Russian sturgeon stocks. Prior to any handling, fish were anaesthetized with 0.6 ml L^{-1} 2 – phenoxyethanol (Merck Co., Darmstadt, Germany).

Flow cytometry

Prior to staining and cell imaging for densitometry, ploidy level of each fish specimen was first verified as relative DNA content in blood cells by means of flow cytometry (Table 1) (Partec CCA I; Partec GmbH, Münster, Germany) using 4′,6-diamidino-2-phenylindole (DAPI) according to Linhart et al. (2006).

Feulgen image analysis

Blood was collected from the caudal vessel into a heparinized syringe according to Pravda and Svobodova (2003), kept at 4°C and processed immediately. All slides were prepared using the 'flame tip' method of smear preparation (Hardie et al., 2002). Standards used were chicken (*Gallus gallus domesticus;* DNA content 2.50 pgDNA nucleus^{-1}) with blood courtesy of the veterinary service of a poultry processing plant in Vodňany, Czech Republic. Diploid and induced triploid tench, *Tinca tinca* (DNA content 2.04 and 3.10 pgDNA nucleus^{-1}, respectively) produced following the protocol of Flajšhans et al. (2004) provided internal standards. All samples were stained using DNA Staining Kit According to Feulgen (Merck Co., Darmstadt, Germany). Feulgen image analysis densitometry following Hardie et al. (2002) was conducted using a 3CCD Sony DXC-9100P camera coupled to an Olympus BX50 microscope (objective 100×), and the OLYMPUS MICROIMAGE v. 4.0 image analysis software package (Olympus Corp., Tokyo, Japan) to measure integrated optical density (IOD) in erythrocyte nuclei, as well as the erythrocyte nuclear area (NA). The RGB colour model software was set to the green channel only, with a maximal 190 pixel intensity; the light intensity calibration was set to Standard Optical Density. Standard curve (IOD vs known DNA content) was generated and used primarily as a check that the stain was accurate across the range of the standards included (Cormier et al., 1993; Flajšhans, 1997; Svobodová et al., 1998; Flajšhans and Vajcová, 2000, Linhart et al., 2001; Hardie et al., 2002). A single primary standard (preferably chicken, DNA content 2.50 pgDNA nucleus^{-1} or tench DNA content 2.04 and 3.10 pgDNA nucleus^{-1}) was used with confidence to calculate genome size.

Mean values and standard deviations (SD) were computed per specimen and per each ploidy level. The effects of fish ploidy on relative DNA content, on the absolute DNA content and NA of erythrocyte nuclei were assessed using ANOVA, which were followed by Tukey's Multiple Comparison Test using STATGRAPHICS v. 5. software. The level of significance was set at $P < 0.01$.

Results

Results on the mean DNA content and mean NA are shown in Table 2. Based on the standard chicken DNA content (2.50 pgDNA nucleus^{-1}) the mean DNA content of diploid and induced triploid *T. tinca* was verified as 2.04 and 3.10 pgDNA nucleus^{-1}, respectively ($P < 0.01$) and used as internal fish standards hereafter. The ratio of DNA content of the induced triploid tench to the diploid one was 1.52, confirming its triploid status.

For both evolutionary tetraploid (i.e. functional diploid) sturgeon species studied, the sterlet and stellate sturgeons, their mean nuclear DNA content in RBCs differed significantly while their NAs exhibited no significant difference, despite high measurement precision with SDs making 7.42 and 5.40% of the mean values for both species, respectively. For both

Table 1
Fish species under study, No. of specimens, their relative DNA contents (mean channel No. and coefficient of variation, c.v. (%) verified using flow cytometry and resulting ploidy levels. Approximately 8 000 cells analyzed per each measurement. Data with same alphabetic superscript not significantly difference at $P < 0.01$

Species	No. of fish analysed (N)	Channel no.	c.v. (%)	Ploidy level	Note
Tinca tinca	20	50.4 ± 2.8^{a}	3.4 ± 0.6	2n	Purebred, internal standard
Tinca tinca	5	75.6 ± 2.5^{b}	3.2 ± 0.8	3n	Induced triploid, internal standard
A. ruthenus	28	101.3 ± 1.8^{c}	3.9 ± 0.9	4n	Purebred
A. stellatus	10	102.6 ± 3.2^{c}	3.4 ± 1.0	4n	Purebred
A. baerii	14	150.25 ± 7.7^{d}	4.5 ± 0.3	6n	–
A. gueldenstaedtii	14	202.5 ± 6.7^{e}	3.5 ± 1.0	8n	Purebred
A. baerii	34	205.4 ± 3.5^{e}	3.6 ± 1.2	8n	Purebred
A. baerii	5	250.9 ± 2.2^{f}	4.0 ± 0.7	10n	–
A. baerii	2	292.8 ± 0.6^{g}	2.7 ± 0.6	12n	Spontaneous triploid
A. gueldenstaedtii	3	292.3 ± 7.0^{g}	3.1 ± 0.2	12n	Spontaneous triploid

Table 2
Fish species under study, evolutionary and functional ploidy levels, absolute DNA content (pg DNA nucleus^{-1}) and nuclear area (NA; μm^2) in erythrocyte nuclei, also results published on their DNA content by other authors.

	Ploidy scale		Nuclear area	DNA content	DNA content by authors	
Species	Evolutionary	Functional	(μm^2)	(pg nucleus^{-1})	(pg nucleus^{-1})	References
Tinca tinca	2n	2n	11.84 ± 0.87	2.04 ± 0.028	2.1 1.94	Wolf et al. (1969) Hafez et al. (1978)
Tinca tinca	3n	3n	15.69 ± 2.22	3.10 ± 0.027	–	–
A. ruthenus	4n	2n	19.27 ± 1.43[a]	3.72 ± 0.024[a]	3.74 4.1	Birstein et al. (1993) Vasil'ev (1999) Zhou et al. (2011)
A. stellatus	4n	2n	19.79 ± 1.07[a]	4.68 ± 0.067[b]	3.74 4.40 4.70	Birstein et al. (1993) Vasil'ev (1999) Zhou et al. (2011) Kafiani et al. (1958)
A. baerii	6n	3n	24.94 ± 1.37[b]	6.30 ± 0.06[c]	–	–
A. gueldenstaedtii	8n	4n	30.09 ± 1.13[c]	7.87 ± 0.068[d]	7.86–7.87 8.40	Birstein et al. (1993) Vasil'ev (1999) Zhou et al. (2011)
A. baerii	8n	4n	29.87 ± 1.80[c]	8.29 ± 0.047[e]	8.29–8.31 8.00	Birstein et al. (1993) Vasil'ev (1999) Zhou et al. (2011)
A. baerii	10n	5n	34.38 ± 1.42[d]	8.99 ± 0.068[f]	–	–
A. baerii	12n	6n	37.82 ± 6.56[de]	12.69 ± 0.433[g]	–	–
A. gueldenstaedtii	12n	6n	41.96 ± 2.60[e]	12.00 ± 0.137[h]	–	–

Data with same alphabetic superscript show no significant differences at $P < 0.01$.

evolutionary octaploid (i.e. functional tetraploid) Siberian and Russian sturgeon species, their mean nuclear DNA content in RBCs differed significantly while their NAs showed no significant difference despite high measurement precision, with SDs reaching 6.03 and 3.76% of the mean values for both species, respectively. For evolutionary hexaploid and decaploid (i.e. functional triploid and pentaploid) specimens found within the Siberian sturgeon stock, their mean nuclear DNA content in RBCs differed significantly as well as their NAs, with high measurement precision with SDs of 5.49 and 4.13% of the mean values, respectively. For evolutionary dodecaploid (i.e. functional hexaploid) specimens of Siberian and Russian sturgeons, their mean nuclear DNA content in RBCs differed significantly along with their NAs, with SDs 17.35 and 6.20% of the mean for specimens of both species, respectively. In these fish, the ratios of their DNA contents to those of usual ploidy level (evolutionary octaploidy / functional tetraploidy) were 1.53 and 1.52, indicating triploidy in both cases.

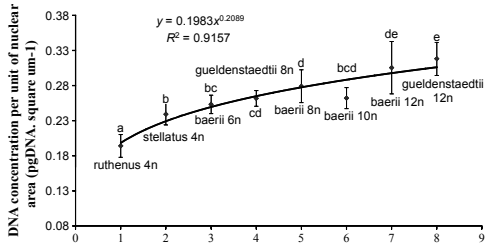

Fig. 1. Relationship between DNA concentration (pgDNA per μm^2 of erythrocyte nuclear area) and ploidy level in sturgeons studied. Data with same alphabetic superscript not significantly different at $P < 0.01$

Results obtained from RBCs of sturgeons in the study (Fig. 1) revealed that the DNA concentration (pgDNA - per μm^2 of RBC nuclear area) was found not to increase linearly with an increasing ploidy level ($r = 0.98$; $R^2 = 0.92$).

Discussion

Comparison of DNA content found in the respective species under study with data published for these species by other authors (Kafiani et al., 1958; Birstein et al., 1993; Blacklidge and Bidwell, 1993; Vasil'ev, 1999; Zhou et al., 2011) is shown in Table 2. All authors used flow cytometry; however, their approaches differed in cell type and / or the fluorescent dye used. Kafiani et al. (1958) analyzed spermatozoa stained with propidium iodide (PI), while most other authors (Birstein et al., 1993; Blacklidge and Bidwell, 1993) working with PI analyzed red blood cells (RBCs). In contrary, Zhou et al. (2011) also used RBCs, but stained with DAPI. Analyzing the information shown in Table 2, it was clear that the data obtained by different authors were not always matching. Perhaps this could be explained by the fact that the PI binds to DNA by intercalating between the bases with little or no sequence preference and that there are shifts in the fluorescence excitation and emission maxima in the red and blue part of the spectrum, respectively (Jones and Kniss, 1987). In contrast, DAPI preferentially stains double-stranded DNA (dsDNA); it appears to associate with AT clusters in the groove. When used according to protocols, DAPI stains nuclei-specifically, with little or no cytoplasmic labeling. The fluorochromes used in

flow cytometry are base pair-specific such that the difference in GC / AT content can affect the measurements when only one stain is used (e.g. Vinogradov, 1994, 1998; Hardie et al., 2002); therefore, for precise measurements and comparisons with other authors, it is necessary to use either both kinds of staining or at least to compare the same type of cells stained with the same dye (T. R. Gregory, pers. comm., 2011). None of the authors dealing with DNA content of sturgeon species under study has ever used the FIA method, however, results obtained with FIA were in good accordance with data established by most other authors (Table 2), with the exception of Zhou et al. (2011).

Another reason for a possible discrepancy could lie in processing the RBCs. Most authors used fresh RBCs for analysis, while Birstein et al. (1993) used fresh RBCs as well as fixed cells. The DNA contents measured in this study exhibited high similarity to those obtained by Birstein et al. (1993).

To the best of our knowledge, very few results on DNA content in sturgeons with ploidy levels higher than evolutionary 8n (functionally 4n) have been published. In fact, only Blacklidge and Bidwell (1993) and Hardie and Hebert (2003, 2004) have published data on DNA content for evolutionary dodecaploid *A. brevirostrum* fishes. Drauch Schreier et al. (2011) recently published data on DNA content for evolutionary decaploid (i.e. functional pentaploid) *A. transmontanus* fishes. The ratio of nuclear DNA content of *A. transmontanus* fishes to those of the usual ploidy level (evolutionary octaploidy / functional tetraploidy) was 1.13–1.34. The ratio of nuclear DNA content of the evolutionary decaploid / functional pentaploid Siberian sturgeon found within the studied stock to those of the usual ploidy level (evolutionary octaploidy / functional tetraploidy) was 1.08. However, due to low numbers of such unique specimens thus far recorded (two fish studied by Drauch Schreier et al., 2011 and five fish in the present study), any conclusions might be difficult.

The present data bring a fresh view on DNA content and NA of *A. gueldenstaedtii* and *A. baerii* exhibiting evolutionary dodecaploidy, as well as evolutionary hexa- and decaploid specimens found within an *A. baerii* stock. According to Fig. 1 it is possible to conclude that the DNA in erythrocyte nuclei appeared to be more and more densely packed with increasing ploidy level. Species of the same ploidy level, however differing in their DNA content, exhibited similar mean erythrocyte nuclear NA, as could be demonstrated on *A. ruthenus* and *A. stellatus* (19.27 and 19.79 μm^2, respectively) with respective mean DNA content 3.72 and 4.68 pgDNA nucleus^{-1}. The same relationship was found for evolutionary octaploid *A. baerii* and *A. gueldenstaedtii* (29.87 and 30.09 μm^2, respectively) with respective to mean DNA content 8.29 and 7.87 pgDNA nucleus^{-1}. Thus the increments 0.19–0.32 pgDNA in DNA content of erythrocytes had no effect on their NA.

In light of the fact that most fisheries genetic laboratories probably were equipped with microscopes and image analysis software, Feulgen image analysis procedure as proposed by Hardie et al. (2002) appeared to be easy accessible, simple to use and a reliable method to estimate the absolute DNA content. The relationship between DNA content and size of RBC nuclei could be considered an important feature that could help molecular and evolutionary biologists interested in characteristics of the genome and, because of the increasing DNA condensation in RBC nuclei with increasing ploidy levels, might help to distinguish specimens > 8n rather than only measuring the dimensions of red blood cell nuclei.

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CHAPTER 4

3-D STRUCTURE, VOLUME, AND DNA CONTENT OF ERYTHROCYTE NUCLEI OF POLYPLOID FISH

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RESEARCH ARTICLE

3-D structure, volume, and DNA content of erythrocyte nuclei of polyploid fish

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Abstract

We have explored the potential relationship between ploidy level, DNA content (pg DNA/nucleus), and dimensional characteristics, such as volume (μm^3), surface area (μm^2), and 3-D structure of erythrocyte nuclei in a series of fish ploidy level models using Feulgen image analysis densitometry, flow cytometry, and confocal laser scanning microscopy. The species were diploid tench (*Tinca tinca*) (2n), Cuban gar (*Atractosteus tristoechus*) (2n), triploid tench (3n), evolutionary tetraploid sterlet (*Acipenser ruthenus*) (4n), evolutionary octaploid Siberian sturgeon (*A. baerii*) (8n), triploid Siberian sturgeon exhibiting dodecaploidy (12n), evolutionary 12n shortnose sturgeon (*A. brevirostrum*), and experimentally obtained sturgeon hybrids that were tetraploid, hexaploid (6n), heptaploid (7n), octaploid, decaploid (10n), dodecaploid and/or tetradecaploid (14n). Increase in ploidy was accompanied by growth of the nucleus and an increase in the number of flattened ellipsoid nuclei with increased transverse diameter. The volume (V_{voxel}) of erythrocyte nuclei, as the sum of voxels calculated from live cells, seems more accurate than volume (V_{axis}) calculated from measuring the major and minor axis, especially at higher and odd ploidy levels. Data of absolute and relative DNA content were in agreement with previously published reports. Species of the same ploidy level, but differing in DNA content, had a similar mean erythrocyte nuclear volume (V_{voxel}), as demonstrated in sterlet and a hybrid of sterlet and beluga (48.3 and 48.9 μm^3, respectively), with a respective mean DNA content of 3.74 and 3.10 pg DNA/nucleus. A similar relationship was found for the ploidy 6n, 10n, 12n. The surface-to-volume ratio decreased non-linearly with increasing ploidy. The DNA in erythrocyte nuclei appeared to be more densely packed with increase in ploidy level.

Keywords: sturgeon; erythrocyte; DNA content; ploidy; nuclear volume; DNA cytometry; 3D confocal analysis

Introduction

The number of chromosomes and amount of genetic material in the cell nuclei of vertebrates has changed during evolution, increasing species diversification (Ohno, 1970; Leggatt and Iwama, 2003). The most significant changes are thought to result from polyploidization, the multiplication of either the entire genome or sets of chromosomes. While uncommon in birds and mammals, polyploidy has repeatedly appeared during the development and diversification of fishes from sharks to the higher teleosts (Leggatt and Iwama, 2003). Sturgeon (Acipenseriformes) and gar (Lepisosteiformes) are ancient groups of ray-finned fishes (extant Actinopterygii). Time estimates of molecular divergence indicate that major clades of Actinopterygii had diversified by the end of the Carboniferous (~300 Ma) (Near and Miya, 2009).

At least three independent polyploidization events have taken place in sturgeon evolution: from the diploid level to the tetraploid, then to octaploid, and finally to dodecaploid (Fontana et al., 2008), but there seem to have been many more (Vasil'ev, 2009). Sturgeon species have been categorized into classes according to chromosome number: (i) species with 120 chromosomes, including all taxa with 110 to 130 chromosomes; (ii) species with 250 chromosomes, including all taxa with 220 to 276 chromosomes; and (iii) *Acipenser brevirostrum* having 360 chromosomes (Blacklidge and Bidwell, 1993; Ludwig et al., 2001; Fontana et al., 2008; Vasil'ev, 2009; Havelka et al., 2011; Zhou et al., 2011). Due to functional genome reduction events,

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some authors (Fontana, 1994; Fontana et al., 1998; Tagliavini et al., 1999; Jenneckens et al., 2000; Ludwig et al., 2001) consider all sturgeon with 120 chromosomes to be functional diploid species, species with 250 chromosomes as functional tetraploid species, and those with 360 chromosomes as functional octaploids. Recently, Fontana et al. (2008) concluded that evolutionary tetraploid, octaploid, and dodecaploid species could be regarded as functional diploid, tetraploid, and hexaploid species, respectively, based on molecular cytogenetic results of fluorescence in situ hybridization (FISH) with DNA probes. To avoid confusion, only the evolutionary ploidy level model will be referenced here. Many biological questions associated with ploidy levels, as well as the mechanisms of polyploidization, are unresolved and may be elucidated by studies of polyploid evolution in groups (Vasil'ev, 2009).

Polyploidy results in larger epidermal, brain and blood cells (Fankhauser, 1955; Licht and Bogart, 1987; Small and Benfey, 1987) and affects cell metabolism and size. Erythrocytes are one of the most reliable cells for quantification of ploidy level because of standardization and ease of sampling. The size of erythrocytes has been proposed as a marker for identifying polyploidy of fish species (Austin and Bogart, 1982; Wolters et al., 1982; Benfey et al., 1984; Garcia-Abiado et al., 1999). Measures of erythrocyte size and volume made from blood smears (Arefjev and Nikolaev, 1991; Palikova et al., 1999; Flajšhans and Vajcova, 2000; Ballarin et al., 2004) might not reflect true dimensions of living cells (Benfey, 1999). However, a strong correlation between erythrocyte size, genome size, and ploidy level has been shown by many authors (Gregory, 2001; Hardie and Hebert, 2003; Flajšhans et al., 2011; Bytyutskyy et al., 2012).

We have studied the potential relationship among ploidy level, genome size, and the following characteristics of erythrocyte nuclei: 3D spatial conformation, volume, and surface area of erythrocyte nuclei of tench (*Tinca tinca*), Cuban gar (*Atractosteus tristoechus*), sterlet (*Acipenser ruthenus)*, Siberian sturgeon *(A. baerii)*, shortnose sturgeon *(A. brevirostrum)*, and several of their hybrids including those of beluga *(Huso huso)* and Russian sturgeon *(A. gueldenstaedtii)*.

Materials and methods

Fish specimens and their processing

Fish samples originating from broodstock at the University of South Bohemia České Budějovice, Faculty of Fisheries and Protection of Waters at Vodňany, Czech Republic, were analyzed. Specimens comprised diploid and triploid tench (produced following the protocol of Flajšhans et al., 2004), Cuban gar, sterlet, Siberian sturgeon, and sturgeon hybrids of varying ploidy levels (Table 1). This study was carried out in strict accordance with the Czech Law n. 246/1992 about "Animal welfare". Protocols have undergone the ethical review process by the University of South Bohemia animal care committee (PP3/FROV/2012; in Czech). All larvae were sacrificed in the water with CO_2, and all efforts were made to minimize suffering.

Ploidy of each parental sturgeon was checked using flow cytometry, and these fishes were crossed and their progeny was obtained. During the larval stage, ploidy level of progeny was also checked by means of flow cytometry. Several ploidy levels were obtained for each group of hybrids. Karyological analyses were made for some of these fishes (Symonova et al., 2010).

After hybrids reached a sufficient size, blood samples were taken from 232 specimens. Prior to handling, fish were anesthetized with 0.6 ml/L 2-phenoxyethanol (Merck Co., Darmstadt, Germany). Blood was collected from the caudal vessel into a heparinized syringe according to Pravda and Svobodova (2003), kept at 4°C, and processed immediately.

Flow cytometry

Prior to staining and cell imaging with confocal laser scanning microscopy (CLSM) and Feulgen image analysis densitometry (FIA), the ploidy level of each specimen was verified with respect to relative DNA content in blood cells by flow cytometry (Table 1) (Partec CCA I; Partec GmbH, Münster, Germany) using 4′,6-diamidino-2-phenylindole (DAPI) according to Linhart et al. (2006). Part of the odd ploidy levels previously seen was not found; absence of expected ploidy levels could be due to the low survival rate of these fishes.

Volume and surface area calculations

The volume of erythrocyte nuclei was obtained by two methods. The first was based on a geometric formula for ellipsoid volume, considering the nucleus as an ideal ellipsoid with equal transverse axes, and is a standard approach for cytological analyses of blood smears:

$$V_{\text{axis}} = \frac{4}{3}\pi\left(\frac{a}{2}\right)\left(\frac{b}{2}\right)^2$$

where V_{axis} = volume of erythrocyte nuclei calculated from fixed dried slides, π = mathematical constant, and a and b = major and minor axes of the erythrocyte nuclei.

A second approach for volume (V_{voxel}) calculation was the application of ImageJ software (Abramoff et al., 2004) and the 3D Object Counter plugin (Bolte and Cordelieres, 2009), which allowed calculation of volume using voxels from stacks of images obtained with CLSM. The same plugin was used to calculate the surface area of erythrocyte nuclei.

Table 1 Fish species studied, coding, number of specimens, relative DNA content (mean channel number and coefficient of variation; c.v. %) verified by flow cytometry, absolute DNA content (pg DNA/nucleus), erythrocytes nuclear volume ($V_{ax\,is}$) verified by FIA densitometry, and the obtained and expected ploidy levels. Approximately 8000 and 100 cells were analyzed for each measurement using flow cytometry and FIA, respectively.

		Flow cytometry			Feulgen Image Analysis Densitometry			
		No. of fish analyzed	Relative DNA content	c.v.	Absolute DNA content	Nuclear volume ($V_{ax\,is}$)	Ploidy level	
Species	Coding	(*N*)	(channel No)	(%)	(pg/nucleus)	(μm^3)	Obtained	Expected
Tinca tinca	*ti2*	20	51.68 ± 1.35^{a}	2.69 ± 0.58	2.04 ± 0.03	24.87 ± 0.66^{a}	2n	2n
Atractosteus tristoechus	*tr2*	5	53.95 ± 3.24^{a}	4.38 ± 0.36	2.79 ± 0.02^{a}	27.97 ± 0.19^{a}	2n	2n
Tinca tinca	*ti3*	10	73.18 ± 2.74^{b}	2.34 ± 0.19	3.1 ± 0.04	27.73 ± 0.3^{a}	3n	3n
A. ruthenus	*ru4*	30	99.77 ± 1.55^{c}	3.88 ± 1.39	3.74 ± 0.01^{b}	56.75 ± 3.06^{b}	4n	4n
A. ruthenus (4n) × *Huso huso* (4n)	*ru4hu4*	25	100.87 ± 1.70^{c}	3.52 ± 0.76	3.1 ± 0.02^{c}	55.66 ± 2.92^{b}	4n	4n
A. ruthenus (4n) × *A. gueldenstaedtii* (8n)	*ru4gu8*	15	152.79 ± 2.47^{d}	3.53 ± 0.35	5.82 ± 0.01^{d}	75.64 ± 3.84^{c}	6n	6n
A. ruthenus (4n) × *A. baerii* (8n)	*ru4ba8*	25	152.78 ± 1.09^{d}	3.36 ± 0.31	6.04 ± 0.02^{e}	75.59 ± 3.93^{c}	6n	6n
A. ruthenus (6n) × *A. ruthenus* (4n)	*ru6ru4*	5	154.18 ± 2.19^{d}	3.26 ± 0.41	5.72 ± 0.15^{f}	74.15 ± 1.86^{c}	6n	5n
A. ruthenus (6n) × *A. ruthenus* (4n)	*ru6ru4*	1	175.4^{e}	3.43	6.4^{g}	77.31 ± 8.27^{c}	7n	5n
A. baerii	*ba8*	40	201 ± 1.35^{f}	3.79 ± 0.94	8.30 ± 0.04^{h}	109.27 ± 5.64^{d}	8n	8n
A. baerii (8n) × *A. baerii* (10n)	*ba8ba10*	14	200.73 ± 1.51^{f}	4.46 ± 0.73	8.34 ± 0.03^{h}	108.68 ± 6.87^{d}	8n	9n
A. baerii (8n) × *A. baerii* (12n)	*ba8ba12*	25	251.72 ± 5.42^{g}	3.84 ± 0.64	8.98 ± 0.03^{i}	132.72 ± 4.79^{e}	10n	10n
A. ruthenus (6n) × *A. gueldenstaedtii* (8n)	*ru6gu8*	2	255.6 ± 6.68^{g}	4.24 ± 0.11	9.44 ± 0.28^{j}	130.51 ± 3.39^{e}	10n	7n
A. baerii (8n) × *A. baerii* (12n)	*ba8ba12*	3	297.54 ± 4.22^{h}	4.12 ± 0.09	12.96 ± 0.06^{k}	164.08 ± 11.03^{f}	12n	10n
A. brevirostrum	*br12*	1	307.86^{h}	3.69	13.02^{k}	154.37 ± 9.16^{f}	12n	12n
A. baerii	*ba12*	1	305.14^{h}	3.54	12.68^{l}	158.12 ± 9.49^{f}	12n	12n
A. baerii (8n) × *A. baerii* (12n)	*ba8ba12*	10	350.16 ± 3.23^{i}	3.39 ± 0.56	15.02 ± 0.04^{m}	178 ± 7.55^{g}	14n	10n

Data with the same superscript letter do not differ significantly at $P < 0.01$.

Feulgen image analysis

Slides with blood smears were prepared using the flame-tip method (Hardie et al., 2002). Diploid and induced triploid tench (DNA content 2.04 and 3.10 pg DNA nucleus, respectively) provided internal standards. Samples were stained using a DNA staining kit according to Feulgen (Merck Co., Darmstadt, Germany). Feulgen image analysis densitometry, following Hardie et al. (2002), was conducted using a 3CCD Sony DXC-9100P camera coupled to an Olympus BX50 microscope (objective 100×) and the Olympus Microimage v. 4.0 image analysis software package (Olympus Corp., Tokyo, Japan) to measure integrated optical density (IOD) in erythrocyte nuclei. The RGB color model software was set to the green channel only, with a maximal 190 pixel intensity. The light intensity calibration was set to Standard Optical Density. A standard curve (IOD vs. known DNA content) was generated and used primarily to confirm that the stain was accurate across the range of the standards included (Cormier et al., 1993; Flajšhans, 1997; Svobodova et al., 1998; Flajšhans and Vajcova, 2000; Hardie et al., 2002).

Confocal laser scanning microscopy

Erythrocyte nuclei were stained with nuclear dye DRAQ5 and fixed in a thermoreversible mountant for live cells CyGEL (both from Biostatus Ltd., UK) according to manufacturer instructions. The dye was diluted in the gel before the cells were added, and stained for 1 h on ice.

Stained nuclei were examined using an upright CLSM (Olympus Fluoview) and Fluoview software. Image stacks of individual erythrocyte nuclei were obtained at excitation wavelength of 568 nm (Kr–Ar laser, operating power 5 mW) with UPlanApo 100× objective in two channels. In the first, the transmitted light image was recorded. Emissions within the range of 585 nm–IF nm in the second channel were detected using a BA585IF filter. The pinhole was 150 nm. The *z*-stacks of confocal images of nuclei were obtained with a 0.20 μm step. Each image stack was processed using ImageJ (1.44l) software (Abramoff et al., 2004). During analysis dividing cells and immature erythrocytes were excluded. The following plugins were used: Bio-Formats Importer for reading life science image file formats, MicroSCoBioJ to create and visualize 3D models, and the 3D Object Counter for calculating surface area and volume of each erythrocyte nuclei (Bolte and Cordelieres, 2009).

Statistical analysis

Mean values and standard deviations (SD) were computed for all studied specimens. The effects of fish ploidy on volume, nuclear surface, and the relative and absolute DNA content of erythrocyte nuclei were assessed using ANOVA

Table 2 Fish species, number of fish and cells analyzed, erythrocyte nuclear volume (V_{voxels}), surface area verified by CLSM, and the resulting ploidy levels.

Confocal laser scanning microscopy

Species	No. of fish analyzed (*N*)	No. of cells analyzed (*N*)	Nuclear volume (V_{voxels}) (μm^3)	Nuclear surface (μm^2)	Ploidy level
Tinca tinca	3	49	27.1 ± 4.4^{a}	84.4 ± 17.9^{a}	2n
Atractosteus tristoechus	1	39	$30.3 \pm 5.5^{a,b}$	83.6 ± 14.7^{a}	2n
Tinca tinca	1	19	$42.0 \pm 3.7^{a,b}$	117.6 ± 9.5^{a}	3n
A. ruthenus	5	246	48.3 ± 6.8^{b}	$114.9 \pm 14.3^{a,b}$	4n
A. ruthenus (4n) × *Huso huso* (4n)	4	239	48.9 ± 6.4^{b}	$121.0 \pm 19.2^{a,b}$	4n
A. ruthenus (4n) × *A. gueldenstaedtii* (8n)	4	239	73.7 ± 5.6^{c}	$153.8 \pm 14.0^{b,c}$	6n
A. ruthenus (4n) × *A. baerii* (8n)	4	85	79.0 ± 14.0^{c}	$179.8 \pm 32.1^{c,d}$	6n
A. ruthenus (6n) × *A. ruthenus* (4n)	5	155	79.3 ± 13.4^{c}	$171.0 \pm 28.4^{c,d}$	6n
A. ruthenus (6n) × *A. ruthenus* (4n)	1	19	$93.2 \pm 4.3^{c,d}$	$188.9 \pm 11.0^{c,d,e}$	7n
A. baerii	7	212	97.2 ± 11.4^{d}	$203.0 \pm 27.5^{d,e}$	8n
A. baerii (8n) × *A. baerii* (10n)	1	13	$113.6 \pm 18.1^{d,e}$	$241.2 \pm 41.4^{e,f}$	8n
A. baerii (8n) × *A. baerii* (12n)	4	235	$130.4 \pm 13.3^{e,f}$	248.7 ± 28.2^{f}	10n
A. ruthenus (6n) × *A. gueldenstaedtii* (8n)	2	51	$128.6 \pm 11.0^{e,f}$	$234.1 \pm 26.2^{e,f}$	10n
A. baerii (8n) × *A. baerii* (12n)	1	76	$139.8 \pm 14.1^{e,f}$	$271.0 \pm 27.1^{f,g}$	12n
A. brevirostrum	1	10	$136.3 \pm 13.1^{e,f}$	$270.3 \pm 58.0^{f,g}$	12n
A. baerii	1	62	148.3 ± 12.8^{f}	$280.6 \pm 22.9^{f,g}$	12n
A. baerii (8n) × *A. baerii* (12n)	1	53	182.3 ± 30.4^{g}	325.8 ± 49.0^{g}	14n

followed by the Tukey's Multiple Comparison Test with the use of Statgraphics v. 5. software. The level of significance was set at $P < 0.01$.

Results and discussion

During hybridization, we had a unique opportunity to study the series of increasing ploidy levels and assess their effect on nuclear size, volume, and DNA content in fishes. Approximately 8000 cells were analyzed from each specimen using flow cytometry and 100 cells with FIA (Table 1). For CLSM, 1802 erythrocyte nuclei from fish with eight ploidy levels were investigated (Table 2). Novel data on volume, surface, and 3D shape of erythrocyte nuclei in sturgeon and sturgeon hybrids of different ploidy levels, as well as tench and Cuban gar, were obtained. There seem to have been only several reports (Arefjev and Nikolaev 1991; Wlasow and Fopp-Bayat 2011) dealing with nucleus and cell volume in sturgeon species, including Siberian sturgeon (8n), beluga (4n), Russian sturgeon (8n), and their hybrids, but no information considering other sturgeon ploidy levels.

Rise in ploidy levels was accompanied by growth of the nuclei and an increased number of more flattened ellipsoid nuclei with increased transverse diameter in higher ploidy levels. All nuclei appeared elliptical with differing dimensions (Fig. 1). Most frequent were typical and flattened ellipsoid nuclei. Less than 1% of all nuclei examined were ideal spheroids. At lower ploidy levels most nuclei had almost ideal ellipsoid shape. It has been shown that triploid individuals possess more elongated nuclei compared to those seen in diploids (Small and Benfey, 1987; Arefjev and Nikolaev, 1991; Wlasow and Fopp-Bayat, 2011). This phenomenon was seen when comparing triploid and diploid tench cell nuclei, but a relationship between odd-number ploidy levels and elongation was not confirmed for ploidy levels higher than triploid.

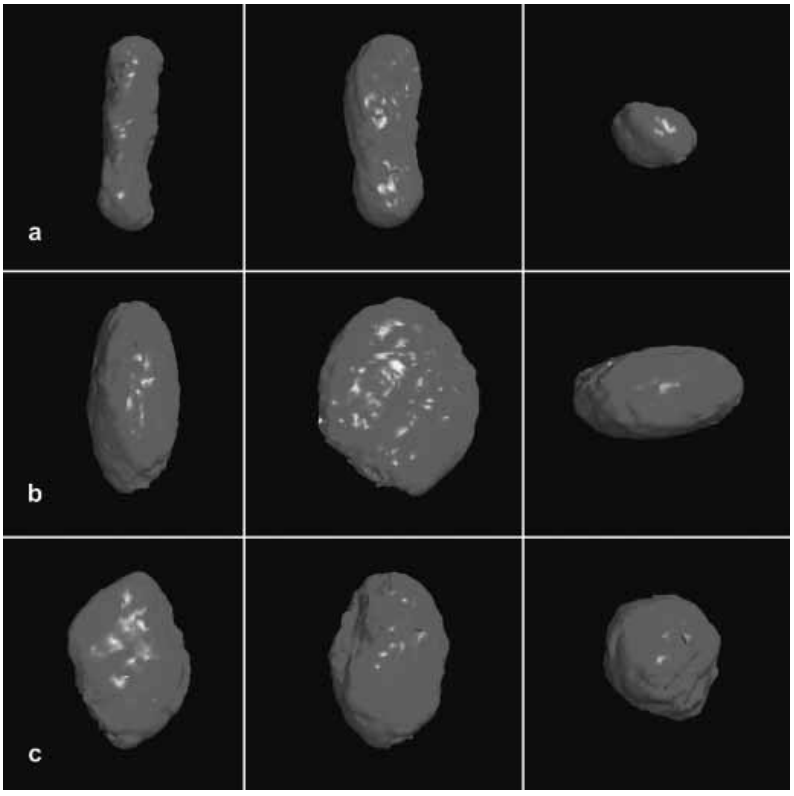

Figure 1 Three-dimensional structure of erythrocyte nuclei visualized using CLSM, ImageJ, and MicroSCoBioJ plugin. (a) Elongate nuclei viewed from different angles; (b) Flattened nuclei viewed from different angles; c) Typical ellipsoid nuclei viewed from different angles.

Mean nuclear volumes (V_{voxel}, V_{axis}) increased with increasing of ploidy levels ($R^2 = 0.9813$; $R^2 = 0.9875$, respectively). Several authors reported finding no significant differences between living and fixed cells with respect to the dimensions of the major and minor axes of erythrocyte nuclei (Ballarin et al., 2004; Flajšhans et al., 2011). Erythrocyte nuclear volume (V_{voxel}) as the sum of voxels, calculated from a living cell fixed with thermoreversible mountant (Table 2) slightly differed from volume (V_{axis}) calculated using the geometric formula (Table 1). It was traditionally used for calculation of fish erythrocytes or erythrocyte nuclei volume from fixed dried samples, using major and minor axes dimensions of the cell or nucleus, obtained from 2D cytometry imaging (Benfey et al., 1984; Nai-Hsien et al., 1993). The highest difference between V_{axis} and V_{voxels} was found in triploid tench ($27.73 \pm 0.3\ \mu m^3$ and $42.0 \pm 3.7\ \mu m^3$, respectively), heptaploid sterlet ($77.31\ \mu m^3$ and $93.2 \pm 4.3\ \mu m^3$, respectively) and dodecaploid sturgeons (e.g. shortnose sturgeon $154.37 \pm 9.16\ \mu m^3$ and $136.3 \pm 13.1\ \mu m^3$, respectively). The explanation of inequality between V_{voxels} and V_{axis} might be seen in increasing number of flattened ellipsoid nuclei in dodecaploid and higher ploidy levels. Flattened ellipsoid nuclei did not have equal transverse axes. This should be seriously considered while using geometrical formula of typical ellipsoid nuclei in high ploidy levels. V_{axis} did not take into account the more complex shape of erythrocyte nuclei, while V_{voxels} included details of shape and surface curvature. This was the main reason why V_{voxel} was considered to be more accurate, especially in the case of high and odd- ploidy levels.

Polyploidisation was associated with increased cell nuclear DNA content and, hence, enlargement. Data of absolute and relative DNA content were in agreement with previously published reports (Bytyutskyy et al., 2012). Sturgeon erythrocytes nuclei have an ellipsoid shape and denser DNA packaging with increasing in ploidy level, but there was linearity between nuclear area and DNA content, which provided a constant DNA concentration as in mouse liver round nuclei hepatocytes (Caspersson, 1937), with regard to the idea that denser DNA packaging may influence form of nuclei. Bytyutskyy et al. (2012) reported unusual ploidy levels in sturgeon (hexa-, deca- and dodeca-ploidy) and described the relationship between DNA content and cell size in 2D. In this study, a similar relationship was found for live erythrocyte nuclei using 3D imaging, confirming the hypothesis of denser DNA packaging at higher ploidy levels.

Species of the same ploidy level; however, different in DNA content led to similar mean erythrocyte nuclear volume (V_{voxels}), as was demonstrated in sterlet and a sterlet and beluga hybrid ($48.2\ \mu m^3$ and $49.5\ \mu m^3$, respectively) with respective mean DNA content of 3.74 ± 0.01 and 3.1 ± 0.08 pg DNA/nucleus. The same relationship was found for ploidy levels such as hexaploid, octaploid, dodecaploid, and decaploid (Tables 1 and 2). Differences of $0.06 - 0.64$ pg DNA/nucleus in erythrocyte DNA content showed no effect on V_{voxels}. The highest erythrocyte nuclear volume and DNA content was found in a 14n hybrid of an 8n and a 12n Siberian sturgeon ($182.3 \pm 30.4\ \mu m^3$ and $178 \pm 8.64\ \mu m^3$ for V_{voxel} and V_{axis}, respectively, with mean DNA content 15.02 ± 0.04 pg DNA/nucleus). There was no clear relationship between DNA content and chromosome number for many organisms (Gregory, 2005a, b). There are many examples when two species

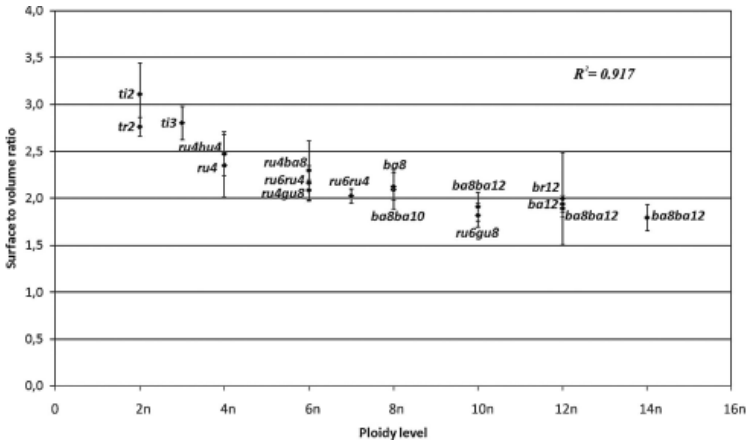

Figure 2 Relationship between surface-to-volume (V_{voxel}) ratio of erythrocytes nuclei and ploidy level in tench, Cuban gar, sturgeon, and sturgeon hybrids.

from the same genus have the same chromosome number and nuclear volume, but different DNA content (Mable et al., 2011). That is why growth of nuclear volume with increase in ploidy level correlates more with chromosome number rather than with DNA content.

Estimated surface-to-volume ratios for species of various ploidy levels showed that, with increasing ploidy levels, surface-to-volume ratio decreases as surface expands more slowly than volume ($R^2 = 0.917$; Fig. 2). At octaploidy and above, the ratio remained stable. It is possible to conclude that nuclear volume and surface-to-volume ratio changes non-linearly with increasing ploidy level. Ploidy levels above the octaploidy have denser DNA packaging with increasing in ploidy level.

In sturgeon species, information about volume or area of RBC can allow easy determination of specimen ploidy level rather than only measure the dimensions of red blood cell nuclei. Dried fixed or living RBC samples can be used for 2D cytometry imaging techniques. Nevertheless, in the case of dodecaploidy and higher ploidy levels, as well as for odd-ploidy levels, it is better to work with living cells and z-axes due to more complicated shape of erythrocytes nuclei, in which it is not possible to describe by normal geometric formula for ellipsoid volume. Changes in erythrocyte nucleus shape and volume, associated with increased DNA density in RBC nuclei, may also help to shed light on the packaging of DNA within the nucleus. With new data on DNA content and volume of tench, Cuban gar, sturgeon and sturgeon hybrids erythrocyte nuclei, we can expand the genome size database and make calculations of the financial and labor costs involved in complete genome sequencing projects.

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CHAPTER 5

GENERAL DISCUSSION

Genome size investigation in sturgeon species

One of the first reports on sturgeon genome size was made by Mirsky and Ris (1951) for *Acipenser sturio*. Since that time big number of studies on sturgeon genome size and/or chromosome number have been done (Table 1). Such huge interest to sturgeon genetics was caused primarily by their evolutionary age and genome evolution due to multiple genome duplication events (Ludwig et al., 2001). All observed pure sturgeon species can be easily divided on three groups: first with ~ 4pgDNA.nucleus^{-1}; second with ~ 8pgDNA.nucleus^{-1}; and third with ~ 13pgDNA.nucleus^{-1}. Present studies (Chapter 3, 4) extent genome size data of pure species as tetraploid *A. ruthenus, A. stellatus, Huso huso, Polyodon spathula* (4n), octaploid *A. baerii, A. gueldenstaedtii* (8n)*;* dodecaploid *A. brevirostrum* (12n). Observed DNA content from these species showed slight discrepancy with results published by other authors (Table 1). Most probable reason of discrepancies in the results observed by different authors can be due to various methodologies, cell types and/or the fluorescent dyes used. Two most commonly used methods for genome size determination are flow cytometry (FC) and Feulgen image analysis densitometry (FIAD), results from this methodologies are usually corresponding. While using FC, it is necessary to use either both commonly used fluorescent dyes, 4', 6-diamidine-2-phenylindole (DAPI) and propidium iodide (PI) or at least to compare the same type of cells stained with the same dye to avoid errors in measurements. Other point is that DAPI preferentially stains double-stranded DNA (dsDNA); it appears to associate with AT clusters in the groove (Vinogradov, 1994, 1998; Hardie et al., 2002). In contrast, PI binds to DNA by intercalating between the bases with little or no sequence preference (Jones and Kniss, 1987). This makes PI more suitable for absolute DNA content measurements. In case of FIAD, it is necessary to pay more attention to additional sources of error like efficacy of dyes, age of slides that has significant effect on the intensity of staining, cell types which may have different level of DNA compaction (Hardie et al., 2002). Some of listed FIAD problems are possible to solve using well known standards. Therefore widely spread freshwater fish as tench was tested and proposed as international standard (Chapter 2) for European aquaculture (Flajšhans et al., 2010), which may be more suitable than commonly used chicken standard that is usually unavailable in the fish farms. Moreover, the ease to induce triploidy in tench and possibility to verify the linearity of DNA content increment in diploid and triploid tench contributed to suitability of tench as standard. In order to check the possibility of using diploid and triploid tench as an international standard, different staining preparation conditions and different measurement systems were used. All three approaches (Chapter 2) resulted in highly similar mean C-values and were found in accordance with previous published data (Vendrely and Vendrely, 1952; Hafez et al., 1978; Flajšhans et al., 2004; Linhart et al., 2006) on tench genome size. Results confirmed that tench can be easily used as standard for identifying ploidy level and/or DNA content in fish.

Sturgeon hybrids

Situation with sturgeons genome is complicated because they can create autopolyploids *de novo*, i.e. in each generation and to hybridize interspecifically or even intergenerically inside the groups of species of the same ploidy level, as well as between groups of species of different ploidy levels, whilst almost all autopolyploids and some hybrids can be morphologically indistinguishable from pure species. For this study, there was a unique opportunity to utilize fish of various ploidy levels, that have been established experimentally for the Czech Science

Foundation grant No. 523/08/0824 (Symonová et al., 2013) and kept alive to study the series of increasing ploidy levels and assess their effect on nuclear size, volume, and DNA content. Present studies (Chapter 3, 4) represent data on nine different sturgeon crosses and spontaneous triploids: *A. ruthenus* (4n) *x Huso huso* (4n) that were defined as tetrploids (4n), *A. ruthenus* (4n) *x A. gueldenstaedtii* (8n) that were defined as hexaploids (6n), *A. ruthenus* (4n) *x A. baerii* (8n) that were defined as hexaploids (6n), *A. ruthenus* (6n) *x A ruthenus* (4n) where were found hexaploids (6n) and one heptaploid (7n) fish, *A. baerii* (8n) *x A. baerii* (12n) where were found decaploid (10n), dodecaploid (12n) and tetradecaploid (14n) individuals, *A. baerii* (8n) *x A. baerii* (10n) that were defined as octaploids (8n), *A. ruthenus* (6n) *x A. gueldenstaedtii* (8n) that were defined as decaploids (10n), dodecaploid *A. baerii* (12n), dodecaploid *A. gueldenstaedtii* (12n). Two hybrids showed different results regarding their ploidy levels. Hybrids between *A. ruthenus* (6n) *x A. ruthenus* (4n) were analyzed during the larval stage and their ploidy was checked using FC. Results of their relative DNA content showed that most of them were pentaploids (5n), but due to the low survival rate in this fishes, almost all of them died, and only six individuals reached sufficient size for blood sampling. As a result there was found five evolutionary hexaploids (6n) and one heptaploid (7n) fish. The occurrence of hexa- and/or heptaploidy in the progeny might be due to fertilization of the aneuploid *A. ruthenus* (6n) ova with spermatozoa of the tetraploid *A. ruthenus* (4n). However, these events require further study. Another hybrid between *A. baerii* (8n) *x A. baerii* (12n) showed quite good survival rate. It was found that most of fishes in this group were decaploids (10n), only three fishes were dodecaploids (12n) and ten fishes were recognized as tetradecaploids (14n). The most plausible mechanism of tetradecaploidy (14n) occurrence supposed to be spontaneous duplication of maternal chromosome set in oocytes, resulting in evolutionary octaploid (8n) level of ovulated oocytes followed by fertilization with a hexaploid (6n) spermatozoon of the dodecaploid (12n) male, in the evolutionary polyploidy scale (Havelka et al., 2014, in preparation), but occurrence of dodecaploid (12n) fishes in this group still remains unclear. Similar idea could explain occurrence of decaploidy (10n) in hybrid between *A. ruthenus* (6n) and *A. gueldenstaedtii* (8n).

Sturgeon cell/nuclear size in 2-D and 3-D

Rise in ploidy level usually correlates with cell/nuclear size. The consequences of nuclear/cell size growth sometimes can influence oxygen transport (hemoglobin concentration), level of protein production, metabolism and other cell/nuclear characteristics (Commoner, 1964; Gregory, 2001; Ballarin et al., 2004). There have been only several reports (Arefjev and Nikolaev, 1991; Wlasow and Fopp-Bayat, 2011; Beyea et al., 2005) dealing with nucleus and cell volume in sturgeon species, including *A. baerii, Huso huso, A. gueldenstaedtii, A. brevirostrum,* and their hybrids, but no information considering other sturgeons. Investigations of different geometrical characteristics were usually performed from dried blood smears using 2-D image analysis. Later, the idea that blood smears did not reflect true dimension of erythrocytes was described in details (Ballarin et al., 2004; Flajšhans et al., 2011). In these studies, dimension of the major and minor axes of erythrocytes and their nucleus was observed from live cells under fluorescent microscope and from dried blood smears under light microscope, as a result there was found no significant difference between geometrical characteristics of dried and live cells conditions. In further study (Chapter 4) live and dried cells were investigated in order to find any difference between 3-D live cells conformation observed using confocal laser scanning microscopy and dried blood cells smears observed under light microscopy using 2-D image analysis technique. As a result significant difference between nuclear volume calculated from dried cells (Benfey et. al, 1984; Nai-Hsien et al., 1993) and volume calculated using

voxel approach (Bolte and Cordelieres, 2009) from live cells was found only in evolutionary high and odd- fish ploidy levels. Volumes that were calculated using voxel approach included more details of shape and surface curvature which seemed to be more complicated in case of odd- and high ploidy levels. The 3-D shape measurements of sturgeon erythrocyte nuclei of different ploidy levels showed that all nuclei appeared elliptical with differing dimensions. At lower ploidy levels most nuclei had almost ideal ellipsoid shape. It has been shown that triploid individuals possessed more elongated nuclei compared to those seen in diploids (Small and Benfey, 1987; Arefjev and Nikolaev, 1991; Wlasow and Fopp-Bayat, 2011). However this elongation was not confirmed in other odd- ploidy levels. Most frequent were typical and flattened ellipsoid nuclei. Flatten ellipsoid nuclei did not have equal transversal axes. This should be seriously considered while using geometrical formula of typical ellipsoid nuclei in high ploidy levels. Less than 1% of all nuclei examined were ideal spheroids. In case of high ploidy levels there was found slight increase in number of flattened ellipsoid nuclei hence this could be the conformation regarding the influence of denser DNA packaging on shape of nucleus, but unfortunately much more investigation in higher ploidy levels needed to confirm this hypothesis. Estimated surface-to-volume ratios for species of various ploidy levels showed that, with increasing ploidy levels, surface-to-volume ratio decreased, as surface expanded more slowly than did the volume ($R^2 = 0.917$). At octaploidy and above, the ratio remained stable. Nuclear volume and surface-to-volume ratio changed non-linearly with increasing ploidy level. Ploidy levels above the dodecaploidy have denser DNA packaging with increasing in ploidy level, confirming the results obtained previously (Chapter 3). It is also necessary to mention that difference in volume and/or surface area of RBC nuclei is big enough for determination of sturgeon ploidy level rather than only to measure the dimensions of red blood cell nuclei.

Sturgeon genome size and cell/nuclear size relationship

Increase in ploidy levels was always accompanied by growth of DNA content, chromosome number and nuclear/cell size. The number of chromosomes and amount of genetic material in the cell nuclei has changed most commonly due to polyploidization, the multiplication of either the entire genome or sets of chromosomes, during evolution, increasing species diversification (Ohno, 1970; Leggatt and Iwama, 2003). There was found no clear relationship between DNA content and chromosome number for many organisms (Gregory, 2005; Gregory, 2014). There are a lot of examples when two species from the same genus have same chromosome number and nuclear volume but different DNA content (Mable, 2011). That is why growth of nuclear volume with increase in ploidy level correlates more with chromosome number rather than with DNA content. Relationships between ploidy level and cell/nuclear size have been well established in context of diploid-triploid-tetraploid fishes by number of authors (Sezaki and Kobayasi, 1978; Wolters et al., 1982; Benfey et al., 1984; Small and Benfey, 1987; Arai et al., 1991; Flajšhans et al., 2011).The relationship between genome and nuclear size in variety of polyploid sturgeons models was shown (Chapter 3), bringing first conformation of denser DNA packaging in erythrocyte nuclei with increasing in high ploidy levels. Both 2-D and 3-D imaging showed no significant difference in case of erythrocyte nuclear volume/surface area of the same ploidy level sturgeons, however there was found visible differences in mean DNA content in case of evolutionary tetraploid, hexaploid, octaploid, decaploid and dodecaploid fishes. The highest erythrocyte nuclear volume and DNA content was found in evolutionary 14n hybrid of an 8n and a 12n Siberian sturgeon (volume ~ 180 μm^3 with mean DNA content 15.02 ± 0.04 pg DNA.nucleus^{-1}). It seems that the "nucleotypic theory", according to results mentioned above, gives the most plausible explanation of the relationship between genome

size and cellular/organismal features. On a certain level, DNA content and cell size are causally related, due to the physical impossibility of containing very large genomes within small cells (Gregory, 2005; Gregory, 2014). According to the results published in chapter 3 and 4, the DNA content increments 1.58 pg DNA.nucleus^{-1} that was found in *A. stellatus* (4n) and hybrid between *A. ruthenus* x *H. huso* exhibiting tetraploidy, 0.58 pg DNA.nucleus^{-1} increment that was found in hybrid between *A. ruthenus* (6n) x *A. ruthenus* exhibiting hexaploidy and hybrid between *A. baerii* and *A. ruthenus* exhibiting hexaploidy, 0.47 pg DNA.nucleus^{-1} increment that was found in hybrid between *A. baerii* x *A. baerii* (10n) exhibiting octaploidy and *A. gueldenstaedtii* (8n), 0.46 pg DNA.nucleus^{-1} increment that was found in hybrid between *A. ruthenus* (6n) x *A. gueldenstaedtii* exhibiting dodecaploidy and *A. baerii* x *A. baerii* (12n) exhibiting dodecaploidy, 1.02 pg DNA.nucleus^{-1} increment that was found between *A. brevirostrum* (12n) and hybrid between *A. gueldenstaedtii* x *A. baerii* (12n) exhibiting decaploidy, did not show any significant influence on thier nuclear volume or nuclear surface. Under the "gene-nucleus interaction model" which was developed by Gregory (2001), it was pointed out that bulk DNA influences the space-filling requirements for cyclins in larger nuclei, and possibly even the influx of regulatory proteins owing to effects on nuclear surface area to volume ratios and/or the arrangement of chromatin within the nucleus. Results on surface-to-volume ratio in species of various ploidy levels (Chapter 4) showed that, with increasing ploidy levels, surface-to-volume ratio decreases as surface expands more slowly than volume. Surface of erythrocyte nuclei increases in non-linear manner with increase in ploidy level. Starting from dodecaploidy we can see stabilizing surface-to-volume ratio. Changes in surface-to-volume ratio could be directly connected to changes in form of the nuclei (number of flatten ellipsoid nuclei starts dominating over the other elliptic shapes). It is possible to hypothesize that sturgeon erythrocyte nuclei proportionally grow in all axis with increase in ploidy level, but after reaching some point near to dodecaploidy two axis do not change as much as the third making nuclei more flatten. This type of changes does not work for all nuclei but for the most of them. Such changes in erythrocyte nuclei and in the entire cell could influence DNA compaction and/or its DNA packaging. Observations showed high DNA concentration in bigger cells and it really seems that "cell size is determined by an interaction of the function of specific genes with the total DNA content of the cell" (Nurse, 1985).

Conclusions

Diploid and triploid tench can be used as international standard for identifying ploidy level and/or DNA content in fish. Using this standard ploidy level, genome size and cell/nuclear geometrical characteristics of pure sturgeons and sturgeon hybrids were analyzed, bringing more information concerning sturgeon's genetics. This data can also expand genome size database in order to help with calculations of the financial and labour costs involved in complete sturgeon's genome sequencing projects (Gregory, 2014).

Apart from the diploid and triploid tench, known ability of sturgeon species to produce interspecific and intergeneric hybrids, as well as to produce spontaneous polyploids in each generation was utilized in order to create model progenies with a series of ploidy levels from tetraploidy to tetradecaploidy, which could be used in the highlighted studies.

There was found no difference between 2-D (dried fixed blood cells) and 3-D (live blood cells) conformation of cell/nuclei in most ploidy levels. Nevertheless, in case of decaploidy and higher ploidy levels as well as for odd- ploidy levels it is better to work with living cells and z-axes due to more complicated shape of erythrocytes nuclei, which is not possible to describe by normal geometric formula for ellipsoid volume. It was also found that number of flatten ellipsoid nuclei increases with rise in ploidy level, starting from dodecaploidy. Subsequent

investigation of octaploids and higher ploidy levels is needed in order to confirm the influence of DNA packaging on shape of nucleus.

Relationships as DNA concentration per unit of nuclear volume and ploidy level, surface-to-volume ratio and ploidy level confirmed that DNA content rise linearly with nuclear size till dodeca- ploidy level. Starting from dodecaploidy to higher ploidy levels, DNA content supposed to be more densely packed or at least rearranged in order to fit new form of nuclei.

Changes that occur in erythrocytes with increase in ploidy level can also affect cell metabolism, hemoglobin concentration, blood flow and other parameters. It seems that there were some physiological changes in fish with high ploidy levels as decaploids and tetradecaploids were more sensitive to injuries and their survival rate also seemed to be lower. High ploidy level fishes usually have smaller size and decreased body growth rate that also possibly correlate with changes in their DNA (person. comment).

This work sheds light on the packaging of DNA at the cellular level, as well as on the relationship between the erythrocyte size, ploidy level and DNA content of sturgeons.

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ENGLISH SUMMARY

Interrelationships between ploidy level, genome size and cell size in series of ploidy level models from 2n to 14n fish

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The ploidy level of diploid and induced triploid tench, *Tinca tinca*, was verified using flow cytometry to determine relative DNA content of 4',6-diamidino-2-phenylindole (DAPI)-stained erythrocyte nuclei. The C-value (haploid nuclear DNA content; pgDNA.nucleus^{-1}) of these same individuals was determined by means of Feulgen image analysis densitometry, in comparison to the chicken standard (*Gallus gallus domesticus*; 1.25 pgDNA.nucleus^{-1}, $P < 0.05$), using three different approaches. Highly similar mean C-values were obtained, thus confirming the possibility of using tench blood as standard in European pond aquaculture for ploidy and DNA content determination in fishes. Feulgen image analysis densitometry (FIAD), flow cytometry (FC) and confocal laser scanning microscopy (CLSM) were used to study the relationship between the DNA content (pgDNA.nucleus^{-1}), nuclear area (μm^2), nuclear volume (μm^3) and 3-D structure of erythrocyte nuclei in a series of fish ploidy level models: diploid tench (*Tinca tinca*) (2n), Cuban gar (*Atractosteus tristoechus*) (2n), triploid tench (3n), evolutionary tetraploid sterlet (*Acipenser ruthenus*) and stellate sturgeons (*A. stellatus*) (4n), evolutionary octaploid Siberian sturgeon (*A. baerii*) and Russian sturgeon (*A. gueldenstaedtii*) (8n), spontaneous triploid Siberian and Russian sturgeons exhibiting dodecaploidy (12n), evolutionary 12n shortnose sturgeon (*A. brevirostrum*), and experimentally obtained sturgeon hybrids that were tetraploid, hexaploid (6n), heptaploid (7n), octaploid (8n), decaploid (10n), dodecaploid (12n) and/or tetradecaploid (14n). Standards used for FIA were blood smears of chicken (2.5 pgDNA.nucleus^{-1}), diploid and induced triploid tench (2.04 and 3.1 pgDNA.nucleus^{-1}, respectively). All ploidy levels were first verified by means of FC. Increase in ploidy was accompanied by growth of the nucleus and an increase in the number of flattened ellipsoid nuclei with increased transverse diameter. The volume (V_{voxel}) of erythrocyte nuclei, as the sum of voxels calculated from live cells, seems more accurate than volume (V_{axis}) calculated from measuring the major and minor axis, especially at higher and odd ploidy levels. Data of absolute and relative DNA content were in agreement with previously published reports. Species of the same ploidy level, however differing in their DNA content, exhibited a similar mean erythrocyte nuclear area, as could be demonstrated on *A. ruthenus* and *A. stellatus* (19.27 and 19.79 μm^2 with a respective mean DNA content of 3.72 and 4.68 pgDNA.nucleus^{-1}) and volume as could be demonstrated on a *A. ruthenus* and hybrid of *A. ruthenus* and *H. huso* (48.3 and 48.9 μm^3 with a respective mean DNA content of 3.74 and 3.10 pg DNA. nucleus^{-1}). Similar relationship was found for the ploidy 6n, 8n, 10n, 12n. The 0.46–1.58 pgDNA increments in DNA content of erythrocytes thus had no effect on their nuclear area/volume. With increasing ploidy level, the DNA concent ration (pgDNA per 1 μm^3 of erythrocyte nuclear volume) as well as surface-to-volume ratio was found not to increase linearly. Nuclear DNA content appeared to be more condensed with an increase of the ploidy level. Observed results deduce properties of whole cell and particularly of the nuclei in series of ploidy levels fishes, adding conformations of nucleotypic hypothesis in context of cell/nuclear size and genome size relationships, as well as taxonomic position of sturgeons.

CZECH SUMMARY

Vztahy mezi úrovní ploidie, velikostí genomu a velikostí buňky v sérii modelů ryb ploidní úrovně od 2n do 14n

Dmytro Bytyutskyy

Ploidní úroveň diploidního a indukovaně triploidního lína obecného, *Tinca tinca*, byla ověřena průtokovou cytometrií s určením relativního obsahu DNA v jádrech erytrocytů barvených 4',6-diamidin-2-fenylindolem (DAPI). C-hodnota (haploidní obsah jaderné DNA; pgDNA.jádro^{-1}) týchž jedinců byla stanovena analýzou obrazu pro denzitometrická měření buněčných jader v preparátech barevných Feulgenovou reakcí (FIAD) ve srovnání se standardem kura domácího (*Gallus gallus domesticus*; 1,25 pgDNA.jádro^{-1}, P < 0,05) třemi postupy. Získány byly vysoce podobné průměrné C-hodnoty potvrzující možnost využít krev lína jako standard v evropské rybniční akvakultuře pro určení ploidie a obsahu DNA u ryb. Obrazová cytometrie FIAD, průtoková cytometrie (FC) a konfokální laserová skenovací mikroskopie (CLSM) byly využity ke studiu vztahů mezi obsahem DNA (pgDNA.jádro^{-1}), plochou jádra (μm^2), objemem jádra (μm^3) a 3-D strukturou jader erytrocytů v sérii ploidních modelů ryb: diploidů lína obecného (*Tinca tinca*) (2n), kostlína kubánského (*Atractosteus tristoechus*) (2n), triploidního lína obecného (3n), evolučně tetraploidního jesetera malého (*Acipenser ruthenus*) a j. hvězdnatého (*A. stellatus*) (4n), evolučně oktaploidního j. sibiřského (*A. baerii*) a j. ruského (*A. gueldenstaedtii*) (8n), spontánních triploidů j. sibiřského a j. ruského vykazujících evoluční dodekaploidii (12n), evolučně dodekaploidního j. krátkorypého (*A. brevirostrum*), a experimentálně získaných jeseteřích hybridů, kteří byli tetraploidní, hexaploidní (6n), heptaploidní (7n), oktaploidní, dekaploidní (10n), dodekaploidní a/nebo tetradekaploidní (14n). Standardy použitými pro FIAD byly krevní nátěry kura domácího (obsah 2,5 pgDNA.jádro^{-1}), diploidní a indukovaně triploidní lín obecný (2,04 a 3,1 pgDNA.jádro^{-1}). Všechny ploidní úrovně byly nejprve ověřeny pomocí FCM. Nárůst ploidní úrovně byl doprovázen zvětšením jádra a zvýšením počtu zploštělých eliptických jader se zvětšeným příčným průměrem. Objem (V_{voxel}) jádra erytrocytu jako suma voxelů počítaná u živých buněk se jeví jako přesnější hodnota než objem (V_{axis}) vypočtený z měření dlouhé a krátké osy jádra, zvláště u vyšších a lichých ploidních úrovní. Údaje o absolutním a relativním obsahu DNA byly v souladu s dříve publikovanými zprávami. Druhy téže ploidní úrovně, jakkoli se lišící obsahem DNA, vykazovaly podobnou plochu jádra erytrocytu, jak lze ukázat na *A. ruthenus* a *A. stellatus* (19,27 a 19,79 μm^2 při průměrném absolutním obsahu 3,72 a 4,68 pgDNA.jádro^{-1}) a podobný objem jádra erytrocytu, jak lze ukázat na *A. ruthenus* a hybridu *A. ruthenus* x *H. huso* (48,3 a 48,9 μm^3 při průměrném absolutním obsahu 3,74 a 3,10 pg DNA. jádro $^{-1}$). Podobné vztahy byly shledány u ploidních úrovní 6n, 8n, 10n, 12n. Nárůst 0,46–1,58 pgDNA v obsahu DNA jader erytrocytů neměl vliv na změnu plochy jádra. Se zvyšující se ploidní úrovní bylo shledáno, že koncentrace DNA (pgDNA na 1 μm^3 objemu jádra erytrocytu) a poměr povrchu jádra k jeho objemu se nezvětšují lineárně. Jaderná DNA je s nárůstem ploidní úrovně stále více kondenzována. Získané výsledky odvozují vlastnosti celých buněk a částečně i jader buněk řady různých ploidních druhů ryb a potvrzují hypotézy o nukleotipickém vztahu velikosti buněk a jejich jader ve vztahu k velikosti genomu a stejně tak taxonomické pozici jeseterů.

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