Fakulta rybářství
a ochrany vod
Faculty of Fisheries
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of Waters

Jihočeská univerzita
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Molecular aspects of interspecific hybridization of sturgeons related to polyploidy and *in situ* conservation

Molekulární aspekty mezidruhové hybridizace jeseterovitých ryb ve vztahu k polyploidii a *in situ* konzervaci

Miloš Havelka

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CONTENT

CHAPTER 1

GENERAL INTRODUCTION

1.1. INTRODUCTION

Sturgeons are of interest genetically and evolutionarily for a variety of reasons. Firstly, sturgeon fishes are supposed to have evolved about 200 million years ago during the Jurassic period (Bemis et al., 1997) and they have not undergone much morphological changes since that time (Gardiner, 1984). Furthermore, the "living fossil" status makes them important for understanding vertebrate evolution in general. These "living fossils" displayed wide range distribution in the northern hemisphere (Fig. 1). From altogether twenty seven acipenseriform species on the Earth, seventeen are supposed to be critically endangered (*Acipenser dabryanus, A. gueldenstaedtii, A. mikadoi, A. naccarii, A. nudiventris, A. persicus, A. schrenckii, A. sinensis, A. stellatus, A. sturio, Huso dauricus, H. huso, Psephurus gladius, Pseudoscaphirhynchus fedtschenkoi, P. hermanni, P. kaufmanni*, *Scaphirhynchus suttkusi*), two species are classified as endangered (*A. baerii*, *S. albus*), four species are vulnerable (*A. brevirostrum, A. ruthenus, P. spathula, S. platorynchus*) and other species are near threatened (*A. medirostris, A. oxyrinchus*) or least concern (*A. fulvescens, A. transmontanus*, IUCN Red list 2012). Moreover, almost all populations of critically endangered sturgeon species are continuously decreasing and extinction of some species seems highly probable (e.g. *P. gladius* or all *Pseudoscaphirhynchus* species).

Several reasons for sturgeon population decline could be identified as follows:

i) The overexploitation of sturgeon population for black caviar

Sturgeon eggs, which are sold as the black caviar, are the most valuable animal product worldwide. Significant increase in international caviar trade during the last centuries led to the dramatic reduction of wild sturgeon populations mainly in Eurasian water bodies (Ludwig, 2008). The situation has been the most critical for *H. huso* in Volga river basin (De Meuleaer and Raymakers, 1996). Although restocking efforts were employed, the harvests of *H. huso* in Caspian Sea were constantly decreasing (Pikitch et al., 2005). Similarly, population abundance of three other main caviar producers in Caspian Sea, i.e. *A. gueldenstaedtii, A. persicus* and *A. stellatus* also declined. Situation became more critical after the brake up of former Soviet Union when illegal harvest and trade rapidly increased with no respect to critical status of sturgeon populations (De Meuleaer and Raymakers, 1996, Pikitch et al., 2005). Concerns about survival of many sturgeon species resulted in listing of all 27 extant acipenseriform species in the Appendices of Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) in 1998. Since that time international trade in all specimens of Acipenseriformes has been regulated under the provision of CITES. Moreover declines in catches led to the development of sturgeon aquaculture farms originally for reintroduction but more recently for caviar production. Saturation of international market with caviar from aquaculture will probably lead to decreasing of prices of black caviar and in combination with releasing programmes, it might help to preserve remaining wild sturgeon populations as was demonstrated in Northern America (Pikitch et al., 2005).

ii) Habitat destruction and dam construction

Degradation of natural environment of acipenseriform species by anthropogenic interventions like river damming, modification or rivers' navigations and water pollution significantly contributed on the decline of abundance of different sturgeon populations (Billard and Lecointre, 2001). Especially, river damming played a major role in this issue.

Acipenseriform species are known for their long migrations. They migrate mostly for reproduction and feeding. Almost all sturgeon species migrate between freshwater and saltwater and only some of them spend their whole lives in freshwater (e.g. *A. baerii* or *A. ruthenus*). Generally, juveniles migrate downstream in order to find enough food while adults undertake a long upstream migration to reach their natural spawning grounds. If the migration routs are blocked by dams, sturgeons are not able to migrate and find enough food or reach their natural spawning grounds which may seriously effected survival of their populations (Billard and Lecointre, 2001). Clear example of this could be found on each continent originally inhabited by sturgeons. In the past, sturgeons from Black Sea used to migrate upstream in the Danube River. Nevertheless, these migrations were interrupted by the Iron Gate Dams completed in 1984 (Kynard et al., 2002) and it considerably contributed on complete disappearance of *H. huso* in the Danube River upstream from the Iron Gate Dams. Similarly, almost all natural spawning ground of three endemic Chinese sturgeon species (*A. dabryanus, A. sinensis, P. gladius*) were lost due to the construction of the Three Gorges Dam on the Yangtze River in China (Wu et al., 2004). Also in the Northern America, sturgeon populations were influenced by dams' construction as was reported by Wirgin et al. (1997). These authors observed smaller population size and lower mitochondrial DNA (mtDNA) diversity of *A. transmontanus* in the Columbia River compared to populations of this species in the Frazer River and they concluded that this fact was caused by presence of dams on the Columbia River.

Pollutions at present play a less important role, but there are some facts that could not be forgotten. Pollution alters fish physiology, especially breeding, it can seriously affect ovarian structure (Moiseeva et al., 1997) and oogenesis (Ruban, 1997) and even results in muscle atrophy (Khodorevskaya et al., 1997) and hepatotoxicity (Geraskin, 1995) in commercially bred sturgeons. High concentrations of PCB in the flesh of *A. oxyrinchus* could cause serious risk for human consumption as was reported by Smith and Clugston, (1997). This might be also the case of high concentrations of DDT and PCB in beluga (*H. huso*) caviar from Black Sea (Wirth et al., 2000).

iii) Losing of genetic integrity of sturgeon species and populations via interspecific hybridization

Natural genetic diversity, which plays important role for surviving of pure species, might be seriously affected by inbreeding and outbreeding depression (Ludwig et al., 2006). Especially outbreeding is a major risk for threatened species due to the small effective population size and its negative impact can be significantly exacerbated by interspecific hybridization (Ludwig et al., 2009). Interspecific hybridization is very common in sturgeon species mainly due to their polyploidy ancestry (Birstein et al., 1997). Moreover, there is no doubt about their threat status and a combination of these facts makes survival of pure sturgeon species very sensitive for interspecific hybridization. Problems of acipenseriform species inferred from interspecific hybridization will be reviewed in detail in the following text.

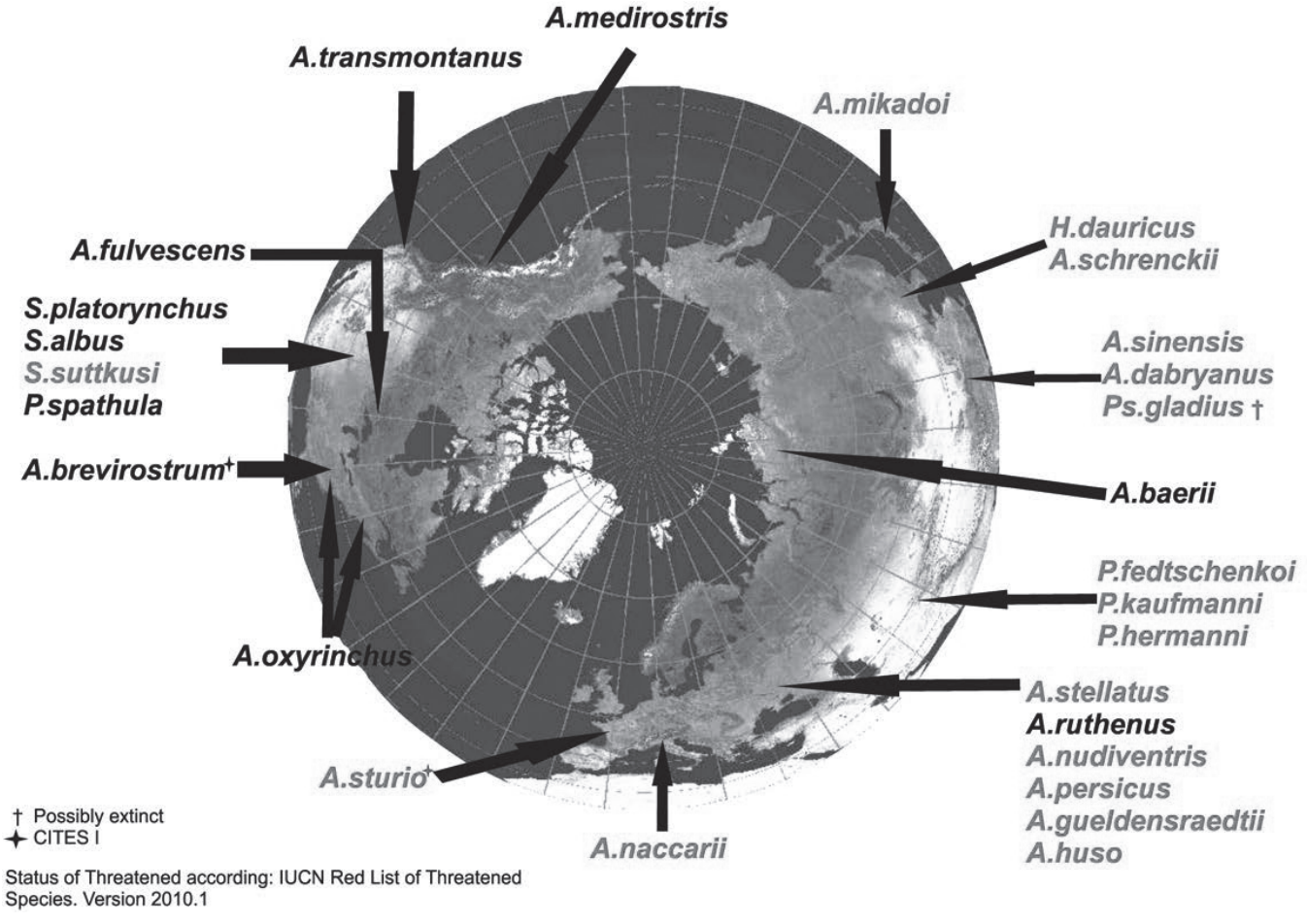

Figure 1. *Distribution of 27 acipenserifom species. Critically endangered species, according to IUCN Red List 2012, are written by grey colour.*

1.2. EVOLUTION OF STURGEON GENOME

Sturgeons are very well known for their polyploidy origin. At least three independent polyploidization events have taken place in their evolution, but in fact there seem to have been many more. Generally, polyploids have been described in many plants and even in some animal species. Polyploidization is now believed to have been the major driving force of plant evolution (Chen, 2007; Soltis and Soltis, 2009), but might be also significant phenomenon in the evolution of fishes (Le Comber and Smith, 2004). The evolutionary implications of polyploidization remain still unresolved (Madlung, 2013) whereas many conflicting options vary from statement that polyploidy being an evolutionary dead end (Arrigo and Barker, 2012) to view that polyploidy have a significant role in speciation and that polyploids being mayor players during evolution (Chen, 2010; Mayfield et al., 2011). Apart from that, origin of polyploids (autopolyploids or allopolyploids) must be seriously considered because differences between them can have significant consequence on their evolutionary success (Madlung, 2013). While autopolyploids arose by genome duplication within same species, allopolyploids by contrast, arose via hybridization of different species and contain two or more distinct genomes. Modern interpretation supposes that hybridization, not genome duplication might be driving force of evolutionary success of some allopolyploids compare to autopolyploids (Wang et al., 2006; Chan, 2007). In contrast Madlung (2013) concluded that it is still impossible to generally infer from available studies if there is any difference in polyploid advantage or disadvantage for autopolyploids compare with allopolyploids. Nevertheless, three major advantages of polyploids that should give them an edge over diploids can be expected. Increasing number of alleles in polyploids could prevent loss of fitness via covering up of deleterious recessive mutations (Gu et al., 2003). Furthermore, heterosis in allopolyploids allows offspring to display transgressive performance compared with its progenitor species (Birchler et al., 2010). Finally, duplicated gene copies can provide slightly varied functions that might potentially increase flexibility and help organisms to cope with the environmental changes (Moore and Purugganan, 2005).

Fishes are the most numerous group of vertebrates, with more then 24 000 species in 57 orders. Nine orders (Acipenseriformes, Atheriniformes, Cypriniformes, Cyprinodontiformes, Lepidosireniformes, Lepisosteiformes, Perciformes, Salmoniformes and Siluriformes) which include majority of fish species (63%) then contain polyploids (Le Comber and Smith, 2004). In addition to that, allopolyploidization does seem to have played important role in speciation of some fish species (Vasiľev, 2009). Clear example of this was given by order Acipenseriformes where several lineage specific genome duplication events, followed by hybridization, occurred (Ludwig et al., 2001; Peng et al., 2007) and sturgeon species with various ploidy levels are still present in most geographic areas of their distribution (Krieger et al., 2008).

Nowadays, several well divided groups of acipenseriform species can be recognized depending on DNA content and the number of chromosomes in their cell nuclei: (i) species with ~ 120 chromosomes and 3.2–4.6 picograms of DNA per nucleus (pgDNA. nucleus^{-1}), (ii) species with number of chromosomes from 240 to 270 and with DNA content 6.1–9.6 pgDNA.nucleus^{-1} (iii) consisting of a single species, *A. brevirostrum*, with 13.1 pgDNA.nucleus^{-1} of DNA (Birstein et al., 1993, Blacklidge and Bidwell, 1993) and a chromosome number of around 360 (Kim et al., 2005). Despite this widely

respected division, ploidy status of Acipenseriformes often remains unresolved with conflicting opinions. Recent investigations suggest two scales of ploidy levels in Acipenseriformes: the 'evolutionary scale', which presumes tetraploid – octaploid – dodecaploid relationships among species, and the 'recent scale', which presumes diploid–tetraploid–hexaploid relationships. In any cases, the common diploid ancestor of all acipenserifom species is generally supposed to have a karyotype of 60 chromosomes (Birstein et al., 1997). Several genome duplication events must have occurred to produce present acipenseriform lineages differing in ploidy levels and chromosome numbers. Timing of these linage specific duplication evens was unclear for a long time since Pang et al. (2007) investigated this topic in detail and brought new insight on this complicated problem. Using Bayesian relaxed molecular clock method of cytochrome b sequences these authors estimated split time between sturgeons and paddlefishes at 184.4 Mya and divergence time between sturgeon Atlantic and Pacific clades about 121 Mya. They also suggested that the genome duplication event in the sturgeon Atlantic linage had occurred 53 Mya while in the Pacific linage 70 Mya. Moreover several recent specific genome duplication events had taken place independently in some sturgeon species, for example in *A. brevirostrum* (ca. within 35.4 Myr) and in *A. mikadoi* (ca. within 9.6 Myr, Pang et al., 2007).

1.3. KARYOLOGY AND PLOIDY LEVELS OF ACIPENSERIFORMES

Order Acipenseriformes displays wide range of chromosome numbers. Especially in family Acipenseridae number of chromosomes varies from ~ 120 to ~ 360 chromosomes (Fontana, 2002). Increasing number of chromosomes is then closely connected with increasing of DNA content in cell nuclei (Zhou et al., 2011; Bytyutskyy et al., 2012). This extensive diversity of chromosome number is also highly supported by common inter-hybridization between pure sturgeon species differing in chromosome numbers which usually resulted in hybrids with intermediate karyotype to those of parental species (Gorshkova et al., 1996). This fact in connection with high number of chromosomes in sturgeon cell nuclei and high number of microchromosomes among these chromosomes can be considered the main reason of complicated interpretation of results of different karyological studies and controversial opinion about ploidy levels of sturgeon species.

The earliest results on sturgeon karyotypes obtained at the beginning of 1960s were affected by technique limitation which did not allow identification of small microchromosomes. For example, first karyotypes of *A. ruthenus* and *H. huso* concerned chromosome number 2n = 60 (Serebryakova, 1972). Nevertheless, already Ohno et al. (1969) indentified 48 michrochromosomes among 112 chromosomes in karyotype of *S. platorynchus.* These authors supposed this species as tetraploid (4n) but they also stated that only observation of diploid (2n) sturgeon species with 60 chromosomes could confirm their hypothesis. Chromosome number 2n = 120 included 48 microchromosomes and gathered in groups of four was assumed by Dingerkus and Howell (1976) for *P. spathula*. Also this species was considered as tetraploid (4n). Almost all authors then encouraged tetraploid status for acipenseriform species with ~120 chormosomes and octaploid status for species with ~ 240–270 chromosomes since Arefjev (1983) expressed debuts about tetraploid condition of ~ 120 chromosome species. This was later supported by findings of

Fontana (1994), Kuzmin (1996), Fontana et al. (1998) and others. These authors considered all sturgeons with ~120 chromosomes to be recent diploids and species with ~ 240–270 chromosomes to be recent tetraploids. Number of chromosomes for different acipenseriform species is summarized in Table 1.

Because chromosome number correlate directly with genome size (Mank and Avise, 2006), the amount of nuclear DNA has been also used for investigation of ploidy relationships among Acipenseriformes. Vialli (1957) detected amount of DNA 3.6 pgDNA.nucleus^{-1} in *A. sturio* and Ohno et al. (1969) found 3.2 pgDNA.nucleus^{-1} in *S. platorynchus*. Twice higher DNA content 6.26 pgDNA.nucleus^{-1} was observed in *A. naccari* by Fontana (1976). More complex studies investigating DNA content of Acipenseriformes were provided by Blacklidge and Bidwell (1993) on seven species from North America and similarly by Birstein et al. (1993) on eight species from Eurasia. Both authors separated sturgeons and paddlefishes into three ploidy groups. The first group was represented by species having DNA content 2.5–4.9 pgDNA.nucleus^{-1} and identified as tetraploids (4n), second group included species with DNA contant 7.8–8.9 pgDNA.nucleus^{-1} and were classified as octaploids (8n). Blacklidge and Bidwell (1993) supposed *A. brevirostrum* as a single member of third group with DNA content 13.08 pgDNA.nucleus^{-1}, in contrast to Birstein et al. (1993) who assigned *A. mikadoi* as an only member of third group with DNA content 13.93 pgDNA.nucleus^{-1}. Moreover, *A. brevirostrum* was considered as evolutionary dodecaploid (12n, Blacklidge and Bidwell, 1993) whereas *A. mikadoi* was expected to have ~ 500 chromosomes referring to evolutionary hexadecaploid (16n, Birstein et al., 1993).

Ploidy status and DNA content of *A. brevirostrum* have reminded constant in literature until now. Kim et al. (2005) identify 372 chromosomes in karyotype of *A. brevirostrum* and Fontana et al. (2008a) determined by means of fluorescent in situ hybridization (FISH) six fluorescent signals in analyzed karyotypes and they subsequently concluded a recent hexaploidy (6n) for this species which exactly corresponded to evolutionary dodecaploid (12n) status suggested by Blacklidge and Bidwell (1993). On the other hand, ploidy level and chromosome number of *A. mikadoi* were discussed many times. In contrast to Birstein et al. (1993) recent studies of *A. mikadoi* karyoptypes revealed about 260–280 chromosomes (Vasliľev et al., 2009, 2010; Vishnyakova et al., 2009; Zhou et al., 2013) and DNA content 8.1–8.3 pgDNA.nucleus^{-1} (Zhou et al., 2011, 2013). Based on recent studies, *A. mikadoi* was classified as evolutionary octaploid (8n) or recent tetraploid (4n). Zhou et al. (2011) also detected high frequencies (45%) of spontaneous triploid individuals among artificially propagated *A. mikadoi* progeny and later on Zhou et al. (2013) confirmed that these specimens had karyotype of 402 chromosomes and DNA content 12.6–13.0 pgDNA.nucleus^{-1}. In addition to that, Zhou et al. (2011) observed few individuals of *A. mikadoi* with DNA content 16 pgDNA.nucleus^{-1}. It is highly probable that four specimens of *A. mikadoi* investigated by Birstein et al. (1993) were these as was later described by Zhou et al. (2011) and it could led to misunderstanding of its ploidy level for a long time.

Some misunderstandings were also found in ploidy levels of *H. dauricus*. First results showed the karyotype of ~ 120 chromosomes (Serebryakova, 1972) and DNA content 3.7–3.8 pgDNA.nucleus^{-1} (Birstein et al., 1993). It referred to evolutionary tetraploid (4n) or recent diploid (2n) status of *H. dauricus*. However, these results were clearly different from recent investigations which revealed ~ 270 chromosomes (Vasiľev et

al., 2009) and DNA content 8.1–8.5 pgDNA.nucleus^{-1} (Zhou et al., 2011) referring to evolutionary octaploid (8n) or recent tetraploid (4n) genome conformation of this species.

The ploidy relationships among extant Acipenseriform groups have been also determined by applying banding patterns in microsatellite markers and investigating of a pattern of microsatellite alleles' inheritance. Microsatellite markers have been developed form several sturgeon species. For example May et al. (1997) developed microsatellite markers for *A. fulvescens*, similarly McQuown et al. (2000) provided large panel of these markers for *S. platorynchus*, King et al. (2001) designed primers for six microsatellite markers in *A. oxyrinchus* and Rodzen and May (2002) developed nine microsatellite markers for *A. transmontanus.* It is evident, that almost all microsatellite markers have been originally developed for North American sturgeon species but later on they were widely used for investigation of ploidy levels and patterns of alleles' inheritance across order Acipenseriformes (e.g. May et al., 1997; McQuown et al., 2000; King et al., 2001; Ludwig et al., 2001; Jenneckens et al., 2001; Welsh et al., 2003 and others).

McQouwn et al. (2000) observed simple diploid allelic bending patterns typical for *Scaphirynchus* species while *A. fulvescens*, *A. transmontanus* and *A. medirostris* displayed allelic bending patterns referring to tetraploid of higher ploidy level. Pyatskowit et al. (2001) examined eleven microsatellite loci in *A. fulvescens* and they observed disomic or tetrasomic allelic bend patterns. All species with ~ 120 chromosomes (*A. stellatus, A. nudiventris, A. ruthenus, A. oxyrinchus, A. sturio* and *H. huso*) investigated by Jenneckens et al. (2001) showed maximally disomic allelic bend pattern at locus Afu 39, while species with ~ 240–270 chromosomes had tetrasomic allelic pattern at that locus. Ludwig et al. (2001) provided complex study investigating ploidy levels of 962 fish from 20 acipenseriform species using six microsatellite markers originally developed for recent tetraploid *A. fulvescens* by May et al. (1997). Ludwig et al. (2001) also included analyses of cytochrom b sequences into their study in order to investigate the evolution of sturgeon genome formation in phylogenetic context. Based on their results, they concluded that species with ~ 120 chromosomes should be classified as functional (recent) diploids, species with ~ 240–270 chromosomes should be classified as functional (recent) tetraploids and with ~ 500 chromosomes as functional (recent) octaploids (*A. mikadoi* was considered to have 500 chromosomes and to be recent octaploid (8n) or evolutionary hexadecaploid (16n) at this time). This could probably cause misinterpretation of data obtained by these authors. Moreover Ludwig et al. (2001) pointed out that not only polyploidization but also genome functional reduction played important role in sturgeon evolution and that those process was nearly finished in species with ~ 120 chromosomes. Finally, they concluded that sturgeon species with ~ 120 chromosomes arose earlier then the others.

Table 1. *Chromosome numbers of different acipenseriform species (according to Havelka et al., 2011).*

Species	Chromosome number	Reference
Scaphirhynchus platorynchus	112	Ohno et al. (1969)
A. nudiventris	116 ± 4	Nowruzfashkhami et al. (2006)
	118 ± 2	Sokolov and Vasiľev (1989)
Huso huso	116 ± 4	Fontana and Colombo (1974)
	118 ± 2	Fontana et al. (1998)
A. sturio	116 ± 4	Fontana and Colombo (1974)
	121 ± 3	Tagliavini et al. (1999)
A. ruthenus	118 ± 2	Fontana et al. (1975)
	118 ± 4	Ráb (1986)
	118 ± 2	Birstein and Vasiľev (1987)
A. stellatus	118 ± 2	Birstein and Vasiľev (1987)
	146 ± 6	Chicca et al. (2002)
Polyodon spathula	120	Dingerkus and Howell (1976)
A. oxyrinchus	121 ± 3	Fontana et al. (2008b)
A. baerii	229 – 240	Fopp-Bayat et al. (2006)
	246 ± 8	Fontana (1994)
	246 ± 10	Fontana et al. (1997)
	249 ± 5	Vasiľev et al. (1980)
A. naccarii	239 ± 7	Fontana and Colombo (1974)
	246 ± 8	Fontana (1994)
	248 ± 4	Fontana et al. (1999)
A. transmontanus	246 ± 10	Fontana et al. (1997)
	248 ± 8	Fontana (1994)
	256 ± 6	Wang et al. (2003)
	271 ± 2	Van Eenennaam et al. (1998)
A. mikadoi	247 ± 33	Vishnyakova et al. (2009)
	262 ± 4	Vasiľev et al. (2009)
	268	Zhou et al. (2013)
A. gueldenstaedtii	249 ± 2	Arefjev and Nikolaev (1991)
	250 ± 8	Birstein and Vasiľev (1987)
	258 ± 4	Fontana et al. (1996)
A. medirostris	249 ± 8	Van Eenennaam et al. (1999)
A. persicus	258 ± 4	Nowruzfashkhami et al. (2000)
A. fulvescens	262 ± 6	Fontana et al. (2004)
A. sinensis	264	Yu et al. (1987)
	264	Zhou et al. (2008)
Huso dauricus	268 ± 4	Vasiľev et al. (2009)
A. brevirostrum	372	Kim et al. (2005)
	372 ± 6	Fontana et al. (2008a)

1.4. INTERSPECIFIC HYBRIDIZATION OF ACIPENSERIFORMES

As it was already mentioned in previous text, interspecific hybridization might cause serious risk for endangered species. Hybridization was identified as the most rapid genetic threat for endangered species with extinction often occurring in less then five generations (Wolf et al., 2001). I addition to that, better growth performance of hybrids might cause replacing of native species and contribute on their extinction (Ludwig et al., 2009). Generally, interspecific hybridization between taxonomically distant vertebrate species differing in chromosome number is rare event due to the incompatibility of their genomes (Arnold, 1997). However, interspecific hybridization was found as not so rare in case of Actinopterygian fish species compare to other vertebrates (Lagler et al., 1962). Already Schwartz (1972, 1981) provided 3 759 references relating to hybridization of fish species wherewith nicely illustrated this feature of fish species. This relative ease with which fishes are able to hybridize is accounted for many factors like external fertilization in water, competition for limited spawning habitats, weak isolating mechanisms, occurrence of parental species in unequal abundances and man-made factors which enhance hybridization. Interesting example of the man-made factor was given by Crego – Prieto et al. (2012). The 70 000 tons of fuel released from sank tanker *Prestige* on the Spanish northwest coast in November 2002. These authors described significant increasing of interspecific hybridization between *Lepidorhombus boscii* and *L. whiffiagonis* after this *Prestige* accident likely due to forced spawning overlap caused by oil spill. Anthropogenic interactions can be also seen as important factors which can enhance sturgeon interspecific hybridization. As was already described in part 1.1., sturgeons undertake long anadromous spawning migrations. If the migration roads are blocked by dams, sturgeons are not able to migrate upstream and reach their natural spawning grounds. Such situation leads to the accumulation of different sturgeon species under the dams and to their spawning in the limited area usually at the same time (Billard and Lecointre, 2001). The probability of interspecific hybridization is then highly increased.

Sturgeons are notoriously known that they hybridize more easily than other fishes (Birstein et al., 1997) and this concern species with the same and/or different ploidy levels. The latter hybridize both in nature (Birstein et al., 1997; Ludwig et al., 2003, 2009; Dudu et al., 2011) and in captivity as well (Arefjev, 1997; Flajšhans and Vajcová, 2000; Zhou et al., 2011 and other). While interspecific hybridization between sturgeons species with the same ploidy level usually result in hybrids owning ploidy same to their parental species, interspecific hybridization between species with different ploidy levels produced hybrids with ploidy level intermediate to those of the parents.

Identification of sturgeon hybrids is still very complicated and only morphological description is not enough to prove that the particular individual is hybrid or not (Ludwig, 2008). Moreover, correct identification of pure acipenseriform species is necessary prerequisite for later identification of hybrids. Only suitable genetic markers and their combination could sufficiently solve these problems (Fontana et al., 2001). Species – specific PCR (Polymerase Chain Reaction) provided useful method for identification of *H. huso* and *A. stellatus* (DeSalle and Birstein, 1996; Birstein et al., 1998), but did not allow differentiation of *A. gueldenstaedtii* from *A. baerii*, *A. naccarii* and *A. persicus* due to the frequent overlapping of their mtDNA

profiles (Birstein et al., 2000; Ludwig et al., 2002). Interspecific nucleotide variation of the mtDNA sequences from nine sturgeon species was investigated by Mugue et al. (2008). These authors provided reliable molecular system for identification of *A. gueldenstaedtii, A. baerii, A. nudiventris, A. stellatus, A. schrenkii, A. ruthenus, H. huso* and *H. dauricus* but similarly to DeSalle and Birstein (1996) and Birstein et al. (1998) their technique displayed limitation for identification of *A. persicus*. Jenneckens et al. (2001) suggested microsatellite locus Afu – 39 (May et al., 1997) as a suitable nuclear marker for *A. stellatus* identification. Restriction Fragment Length Polymorphism (PCR – RFLP) was investigated by Ludwig et al. (2000) as a possible discrimination technique among twenty two acipenseriform species. While seventeen species displayed species – specific differences suitable for their discrimination, no diagnostic substitution were found for the species pair *A. gueldenstaedtii / A. persicus* and for all species from genus *Scaphirynchus* (Ludwig et al., 2000). Also Random Amplified Polymorphic DNA (RAPD) technique was discussed as a suitable method for sturgeon species identification (Comincini et al., 1998; Bermintsev et al., 2001; Gharaei et al., 2005) but this procedure showed some limitation (mainly low reproducibility) for this purpose. All methods, suitable for sturgeons' species identification were summarized by Ludwig (2008). This author recommended sequencing, nested – PCR and PCR - RFLP of mtDNA as the most suitable tool for species identification. Nevertheless, analyses of mtDNA have limitation for hybrids identification due to maternal inheritance.

Number of sturgeon hybrids was constantly increasing in sturgeon aquaculture during last 60 years (Bronzi, 1999), mainly because they were considered to perform better compared to pure species. It led to rapid utilization of hybrids in sturgeon aquaculture and many different sturgeon hybrids were artificially produced (Krylova, 1999). On the other hand, Billard and Lecointre (2001) concluded that sturgeon hybrids usually did not perform better then the best parents and because there has not been given clear demonstration of superiority of sturgeon hybrids, their using in aquaculture is questionable. Moreover escapes of farmed sturgeon hybrids into open waters could cause serious risk of genetic contamination of wild sturgeon population.

Although Ludwig (2008) claimed that natural sturgeon hybrids were rare, several interspecific hybrids, observed in natural conditions, have been already described. Using analyses of mtDNA cytochorm *b* gene, Jenneckens et al. (2000) reported contamination of *A. gueldenstaedtii* with *A. baerii* in Volga River. Similarly Ludwig et al. (2009) observed genotypes and morphotypes of *A. baerii* in Upper Danube. This species has originally inhabited Asian rivers but it became widely preferred in sturgeon aquaculture due to easy handling, fast growing and non-complicated reproduction (Bronzi et al., 1999). Furthermore escapes of this non-native species from aquaculture might have frequently occurred (e.g. Maury-Brachet et al., 2008). This resulted in increasing catches of *A. baerii* in European rivers which strongly correlated with their increasing numbers in European aquaculture (Gesner et al., 1999; Arndt et al., 2002; Holčík et al., 2006; Masár et al., 2006). Ludwig et al., (2009) firstly documented natural reproduction of Siberian sturgeon *A. baerii* outside their natural range in Europe and using combination of mtDNA control region and seven microsatellite loci, they described several interspecific hybrids between exotic *A. baerii* and native *A. ruthenus*. The interspecific hybridization was also observed among species which originally inhabited same area. The clear example was given by

Dudu et al. (2011) from the Black Sea and Lower Danube. These authors used analyses of eight microsatellite markers in combination with three successive statistical analyses (Factorial Correspondence Analysis – FCA, STRUCTURE assignation test and NEWHYBRIDS status determination) and they observed interspecific hybrids and frequent back crosses among four native acipenseriform species in The Danube River and Black Sea (*A. ruthenus*, *A. gueldenstaedtii*, *A. stellatus H. huso*). Similar situation occurred in the Mississippi River where the interspecific hybridization between two native species *S. albus* and *S. platorynchus* was detected by Tranah et al. (2004). Congiu et al. (2001) used Amplified Fragment Length Polymorphism (AFLP) profiles to separate hybrids between *A. naccarii* and *A. transmontanus* from their parental species. Ludwig (2008) suggested this technique as very useful for discrimination of species with a low level of genetic divergence and also for backcrosses screening, but he also strongly recommended inclusion of reference specimens of parental species.

Another important question related to sturgeon interspecific hybrids is their fertility or sterility. Generally in fish interspecific hybrids of distantly related parental species are usually sterile because their chromosomes cannot correctly pair during zygotene stage of meiosis prophase I and such impairment interferes with gonadal development and gametogenesis (Pifferer et al., 2009). Hybrids between sturgeon species with same ploidy levels are generally considered to be fertile while hybrids between sturgeon species differing in their ploidy level are usually supposed to be infertile. Nevertheless, Flajšhans and Vajcová (2000) discussed possible fertility of sturgeon hybrids having intermediate DNA content referring to recent triploids (3n) or evolutionary hexaploids (6n). Using flow cytometry, these authors detected evolutionary pentaploids (5n) and evolutionary heptaploids (7n) individuals among sturgeon aquaculture stocks and they suggested that these individuals might originated from hybridization of evolutionary hexaploid specimen (6n) with evolutionary tetraploid specimen (4n) and evolutionary hexaploid specimen (6n) with evolutionary octaploid specimen (8n) respectively.

A study of sturgeon genetics can show us to what extent the polyploidization events played an important role during the evolution of vertebrates and new results indicate that these events still continue among different sturgeon species. There is still no clear view on the status of sturgeon polyploidy (e.g. paleotetraploidy vs. modern diploidy) even inside the scientific community dealing with this question till now. In addition to that, polyploid status of sturgeon species is significant prerequisite for frequent interspecific hybridization among them. All these facts make studying of sturgeon genetics very complicated on the one hand, but also very interesting on the other. It is also evident that it will require further studies in the future to understand better to genetic background of these pretty polyploids, however, threatened or endangered status of many of acipenseriform species indicates that there may be a limited time left to study these organisms.

1.5. AIMS OF THE THESIS

The main aims of this work were to:

i) Investigate the ploidy levels of different sturgeon species using highly polymorphic microsatellite markers and clarify the ploidy relationships among sturgeon species from different ploidy groups in respect to their evolution via polyploidization events.
ii) Clarify the origin of abnormal ploidy levels and assess if these are autopolyploids or allopolyploids, incurred by reproductive contact of two or more species.
iii) Observe segregation pattern of microsatellite alleles in the course of hybridization of polyploid sturgeon species.
iv) Evaluate the impact of inter-specific hybridization of surgeon species on viability of resulting progeny and possible fertility of sturgeon species with abnormal ploidy levels.

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CHAPTER 2

EXTENSIVE GENOME DUPLICATION IN STURGEONS: NEW EVIDENCE FROM MICROSATELLITE DATA

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Extensive genome duplications in sturgeons: new evidence from microsatellite data

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Summary

Genome duplications and polyploidization events are thought to have played relevant roles in the early stages of vertebrate evolution, in particular near the time of divergence of the lamprey lineage. Additional genome duplications, specifically in ray-finned fish, may have occurred before the divergence of the teleosts. The role of polyploidization in vertebrate genome evolution is a thriving area of research. Sturgeons (order Acipenseriformes) provide a unique model for the investigation of genome duplication, with existing species possessing 120, 250 or 360 chromosomes. In the present study, data from 240 sturgeon specimens representing 11 species were used for analysis of ploidy levels. Allele numbers were assessed at eleven microsatellite loci. The results provide further evidence for functional diploidy, tetraploidy and hexaploidy in species possessing 120, 250 and 360 chromosomes, respectively. The analysis also uncovered novel evidence for functional hexaploidy in the shortnose sturgeon (*Acipenser brevirostrum*). In conclusion, the process of functional genome reduction is demonstrated to be an on-going process in this fish lineage.

Introduction

Polyploidization is a highly dynamic process that plays a major role in the evolution and speciation of fish species. After some hundred million years of speciation the well-established polyploid species, including sturgeons and paddlefishes (Acipenseriformes), are notable. Acipenseriformes evolved at least 200 million years ago; since this time several lineage-specific genome duplication events have occurred (Peng et al., 2007) and species with various ploidy levels are still present in most geographic areas of their distribution (Krieger et al., 2008). Modern interpretation of polyploidy evolution of Acipenseriformes using DNA content (C-value) has contradicted karyological data, according to which the Acipenseriform species can be divided into three groups: group A with approximately 120 chromosomes and 3.2–4.6 picograms (pg) of DNA; group B with approx. 250–270 chromosomes and 6.1–9.6 pg of DNA; and group C consisting of a single species, the shortnose sturgeon, *Acipenser brevirostrum*, with 13.1 pg of DNA (Blacklidge and Bidwell, 1993) and a chromosome number of around 360 (Fontana et al., 2008a).

Whilst karyotype data distinguishes three discrete groups, the DNA contents indicate a continuum over a wide range of values. However, any change in DNA content may reflect both polyploidization events and/or other well-described genetic mechanisms. It is also assumed that evolution of polyploidy in Acipenseriform fishes can be better understood by studying the mechanisms or modes of polyploidization. So far, the ploidy relationships among extant Acipenseriform groups have been determined by applying a variety of techniques such as chromosome counts (Fontana, 2002), banding patterns in microsatellite markers (Ludwig et al., 2001), cell size comparisons and genome size estimations (Birstein et al., 1993).

Despite this array of methods, they are contested in the literature; the polyploidy status of Acipenseriformes therefore often remains unresolved, with conflicting opinions. Recent investigations suggest two scales of ploidy levels in Acipenseriformes: the 'evolutionary scale', which presumes tetraploid–octaploid–dodecaploid relationships among species, and the 'recent scale', which presumes diploid–tetraploid–hexaploid relationships. In any case, the dominant view is that the Acipenseriformes arose by a polyploidization event from a diploid ancestor (2n = 60 chromosomes), with the reestablishment of functional diploidy occurring before the major radiation of the order (Birstein et al., 1997). Additional duplication restricted to members of the genus *Acipenser* have since occurred, and indeed it is believed that higher ploidy levels have evolved several times independently (Ludwig et al., 2001). This process may still be on-going, because triploid individuals can occasionally be found within otherwise diploid species (Blacklidge and Bidwell, 1993). In light of publications to date, sturgeons still offer an ideal opportunity to study the process of polyploidization and secondary diploidization after tetraploidy as an on-going process.

In this paper we used eleven highly polymorphic microsatellite markers to investigate the ploidy relationships of eleven sturgeon species. More specifically, we examined the existence and extent of duplications that have occurred in these species.

Materials and methods

Fish samples and DNA extraction

Sturgeon species investigated in the present study are listed in Table 1. Whole genomic DNA was extracted from fin clips stored in 96% molecular grade ethanol using the NucleoSpin® Tissue DNA extraction kit (Macherey-Nagel, Germany) following recommendations of the manufacturer.

Microsatellite analyses

Following initial testing involving 13 microsatellite markers developed for sturgeon species, 11 markers: Afu 34, Afu 54, Afu 68 (May et al., 1997), Spl 101, Spl 105, Spl 163, Spl 173

Table 1
Origins of samples from 11 extant Acipenseriform species used in this study and three ploidy groups (A, B and C) in the order Acipenseriformes based on chromosome number and/or DNA content reported in previous studies

Species	Common name	Group[b]	No. of chromosomes	DNA content (pg)	Sample size	Geographic origin of sampled species	References
Acipenser nudiventris	Ship sturgeon	A	118 ± 2	3.9	32	Caspian Sea/ Iranian coast	Birstein et al. (1993)
Acipenser stellatus	Stellate sturgeon/sevruga[a]	A	118 ± 2	4.4	22	Aquaculture/Russia	Zhou et al. (2011)
Acipenser ruthenus	Sterlet	A	121 ± 3	4.1	30	Aquaculture/ Danube River	Zhou et al. (2011)
Acipenser oxyrinchus	Atlantic sturgeon	A	118 ± 2	4.55	9	Saint John River/Canada	Fontana et al. (2008b)
Huso huso	Great sturgeon/beluga[a]	A	118 ± 2	3.6	26	Danube River	Fontana (1976)
Acipenser baerii	Siberian sturgeon	B	246 ± 8	8.0	40	Aquaculture/Siberia, Russia	Zhou et al. (2011)
Acipenser gueldenstaedtii	Russian sturgeon/osetra[a]	B	258 ± 4	8.4	35	Aquaculture/Russia	Zhou et al. (2011)
Acipenser persicus	Persian sturgeon	B	258 ± 4	–	21	Caspian Sea/ Iranian coast	Nowruzfashkhami et al. (2000)
Acipenser naccarii	Adriatic sturgeon	B	248 ± 4	6.26	1	Po River, Italy	Fontana (1976)
Acipenser mikadoi	Sakhalin sturgeon	B	247 ± 33	8.2	2	Tumnin River, Russia	Zhou et al. (2013)
Acipenser brevirostrum	Shortnose sturgeon	C	372 ± 6	13.08	22	Saint John River/Canada	Kim et al. (2005)

Group A, evolutionary tetraploid/functional diploid species with ca. 120 chromosomes; Group B, evolutionary octaploid/functional tetraploid species with ca. 250–270 chromosomes; Group C, evolutionary dodecaploid/functional hexaploid species.
[a]Trade name of caviar.
[b]According to Fontana et al. (2008a).

(McQuown et al., 2000), Aox 45 (King et al., 2001), AfuG 54, AfuG 135 (Welsh et al., 2003) and Atr 114 (Rodzen and May, 2002) that consistently amplified PCR products of appropriate size were selected for subsequent analyses. PCR were carried out using the Poor Man's PCR protocol (Schuelke, 2000). Amplifications were performed under the reaction profile: one cycle at 94°C for 3 min, 25 cycles at 94°C, followed by locus-specific annealing conditions (Table 2), and a final extension at 72°C for 10 min. One of the primers within each of the 11 primer sets possessed a 5′ prime end tail (M13, CAG, GODDE, or HILL). During standard PCR, a fluorescently (VIC, PET, FAM, or NED) labelled primer (M13, CAG, GODDE, or HILL) was added to the amplification reaction. The resulting PCR products were run into the ABI 3730XL DNA analyser, and genotypes were scored using GeneMapper v4.1 (Applied Biosystems, TM). Given the polyploid nature of Acipenseriformes the number of alleles detected per individual varied locus by locus. The ploidy level and the degree of genome duplication in each of the species considered in this study were evaluated by examining the maximum number of alleles per individual found at each locus, which should reflect the minimal ploidy level. Maximally two alleles differing in size (diploid allelic band pattern) were expected for diploid locus, four alleles differing in size (tetraploid allelic bend pattern) for tetraploid locus, etc. Low level polymorphism at some loci may theoretically influence the possibility to find the maximal number of alleles within samples; to eliminate this we used large sets of microsatellite markers.

Results

A summary of the ploidy levels inferred from the analysis of the microsatellite data is displayed in Table 3. With few exceptions, seven of the microsatellite loci examined in this study (Afu 34, Afu 54, Spl 101, Spl 105, Spl 163, Spl 173, Aox45) exhibited typical diploid and tetraploid allelic band patterns in species with approximately 120 and approximately 250 chromosomes, respectively. The remaining four microsatellite loci (Afu 68, AfuG 54, AfuG 135, Atr 114) displayed a tetraploid allelic band pattern in species with approximately 120 chromosomes and, in most instances, an octaploid allelic band pattern in species with approximately 250 chromosomes.

An exception to this general band pattern was observed in *A. brevirostrum* for which five loci showed a hexaploid allelic bend pattern (Spl 101, Spl 105, Spl 163, Spl 173, Aox 45), three loci (Afu 68, AfuG 54, AfuG 135) showed evidence of a possible octaploid allelic bend pattern, and twelve distinct alleles were observed at a single locus (Atr 114).

All additional data including allele size ranges and the number of alleles observed at each locus within each analysed individual per species are listed in Table 2.

Discussion

In the present study a set of eleven highly polymorphic microsatellite markers have been used to examine ploidy levels to determine the incidence and extent of duplications in sturgeon genomes. Eleven sturgeon species representing the three ploidy scales suggested by Fontana (2002) were investigated. To best of our knowledge this report provides the most complex study of ploidy levels and genome duplications in sturgeon species examined by microsatellite markers. Our results indicate that, based on the strictly diploid allelic band pattern found at seven microsatellite loci, the species characterized by approximately 120-chromosomes (Group A) could be classified as functional diploid species. Most species with approximately 250–270 chromosomes (Group B), displayed a tetraploid allelic band pattern at seven loci and, as such, could be classified as functional tetraploids. These findings support the work of Fontana (1994) and Ludwig et al. (2001), which were based on nucleoar

Table 2
Microsatellite markers used in this study, their origins, and locus-specific annealing temperatures and range of allele size for each investigated species

Marker	Originally developed for	Tailed	Amplification temperature	*Acipenser nudiventris*	*Acipenser stellatus*	*Acipenser ruthenus*	*Acipenser oxyrinchus*	*Huso huso*	*Acipenser baerii*	*Acipenser gueldenstaedtii*	*Acipenser persicus*	*Acipenser mikadoi*	*Acipenser naccarii*	*Acipenser brevirostrum*
Afu 34	*Acipenser fulvescens*	M13R	57°C	156 (1)	159–168 (2)	159–168 (4)	134–180 (3)	162 (1)	156–168 (4)	156–168 (7)	159–168 (4)	159 (1)	156–162 (2)	162–168 (3)
Afu 54	*Acipenser fulvescens*	HILL	55°C	218–222 (2)	186–214 (7)	190–194 (2)	186–214 (4)	260–291 (4)	190–278 (12)	190–262 (15)	194–246 (10)	198 (1)	222–266 (4)	172–266 (6)
Afu 68	*Acipenser fulvescens*	CAG	57°C	260–268 (3)	120–160 (9)	196–248 (11)	140–176 (7)	156–260 (6)	148–252 (16)	128–258 (22)	160–264 (7)	102–188 (8)	164–216 (4)	120–228 (15)
Spl 101	*S. platorynchus*	M13R	57°C	318–334 (4)	306–330 (7)	294–334 (7)	268–314 (6)	288–294 (2)	306–362 (10)	294–342 (13)	306–346 (9)	328–344 (4)	306–420 (4)	302–334 (6)
Spl 105	*S. platorynchus*	HILL	59°C	146–156 (3)	152–176 (6)	130–164 (7)	–	142–172 (5)	146–168 (7)	130–176 (8)	138–164 (4)	138–184 (9)	152–176 (4)	130–176 (12)
Spl 163	*S. platorynchus*	CAG	57°C	208–240 (4)	190–224 (9)	212–232 (6)	–	204–220 (5)	212–268 (13)	188–268 (11)	188–232 (8)	212–280 (5)	220–256 (3)	192–228 (9)
Spl 173	*S. platorynchus*	M13R	57°C	247–263 (4)	247–296 (7)	241–279 (7)	–	231–259 (4)	241–281 (14)	247–277 (14)	255–269 (8)	271 (1)	263–274 (4)	247–285 (10)
Aox45	*Acipenser oxyrinchus*	CAG	53°C	146–171 (5)	134–167 (9)	137–161 (9)	137–167 (6)	140–149 (4)	137–184 (17)	131–195 (19)	135–184 (12)	149–177 (5)	152–190 (4)	147–195 (15)
AfuG 54	*Acipenser fulvescens* *Acipenser medirostris*	M13R	53°C	257–282 (4)	252–288 (10)	244–300 (9)	240–273 (6)	244–290 (9)	238–286 (15)	238–306 (26)	262–306 (12)	257–290 (8)	244–282 (6)	254–274 (11)
AfuG 135	*Acipenser fulvescens* *Acipenser medirostris*	CAG	53°C	204–268 (8)	196–262 (7)	204–256 (9)	196–224 (5)	196–232 (7)	196–264 (18)	196–268 (19)	200–284 (15)	236–248 (4)	200–256 (6)	196–252 (14)
Atr 114	*Acipenser transmontanus*	HILL	57°C	–	–	224–268 (11)	228–236 (3)	215–263 (9)	212–272 (17)	216–284 (23)	218–272 (15)	206–248 (4)	218–320 (6)	212–258 (22)

In parentheses: Numbers of alleles in analyzed microsatellite loci.

Table 3
Ploidy level as inferred from microsatellite analysis for 11 extant Acipenseriform species examined in this study

	Microsatellite loci examined in this study											
Species	Afu 34	Afu 54	Spl 101	Spl 105	Spl 163	Spl 173	Aox 45	Afu 68	AfuG 54	AfuG 135	Atr 114	Inferred ploidy level
Acipenser nudiventris	2n	2n	2n	2n	2n	2n	2n	2n	4n	4n	–	2n
Acipenser stellatus	2n	2n	2n	2n	2n	2n	2n	4n	4n	2n	–	2n
Acipenser ruthenus	2n	2n	2n	2n	2n	2n	2n	4n	4n	4n	4n	2n
Acipenser oxyrinchus	2n	2n	2n	–	–	–	2n	4n	4n	2n	2n	2n
Huso huso	2n	2n	2n	2n	2n	2n	2n	2n	4n	4n	4n	2n
Acipenser baerii	4n	4n	4n	4n	4n	4n	4n	4n	8n	8n	8n	4n
Acipenser gueldenstaedtii	4n	4n	4n	4n	4n	4n	4n	8n	8n	8n	8n	4n
Acipenser persicus	2n	4n	4n	2n	4n	4n	4n	4n	8n	8n	8n	4n
Acipenser mikadoi	2n	2n	4n	–	4n	2n	4n	8n	8n	4n	4n	4n–8n[a]
Acipenser naccarii	2n	4n	4n	4n	3n	4n	4n	4n	8n	8n	8n	4n[a]
Acipenser brevirostrum	4n	4n	6n	6n	6n	6n	6n	8n	8n	8n	12n	6n

Numbers beneath microsatellite loci indicate allelic band patterns observed for each species at each locus, 2n = diploid allelic band patterns, 4n = tetraploid allelic band patterns, etc, – = no amplification at the locus indicated for that species.
[a]Due to low number of samples used, true ploidy levels could not be detected.

organizing regions (NORs) and microsatellite markers, respectively. Thus, both studies suggested that the species in Group A should be considered as recent diploids while species from Group B should be classified as recent tetraploids.

The results presented here also show evidence for the coexistence of diploid and tetraploid and/or tetraploid and octaploid allelic band patterns in the same genomes of investigated species, as was assumed by Ludwig et al. (2001) who used six microsatellite markers originally developed for *A. fulvescens* (May et al., 1997) and also observed two duplicated loci (Afu 57 and Afu 68) in species with approximately 250 chromosomes. Results of the present study provide a more detailed view of this complicated problem. Compared to the study of Ludwig et al. (2001), we observed four duplicated loci (Afu 68, AfuG54, AfuG135, Atr 114) within eleven of those investigated and show evidence of loci duplication in species with approximately 120 chromosomes as well as in *A. brevirostrum* that was not described by those authors.

Observations of the coexistence of diploid and tetraploid and/or tetraploid and octaploid allelic band patterns in the same sturgeon genome may be ascribed to a secondary functional diploidization event (Tagliavini et al., 1999; Fontana et al., 2008b). In allotetraploids, for example, when the original hybridization event involves very similar genomes, different chromosomes are likely to display different degrees of homology, with some being totally homologue and others different enough to cause the distinction of the parental species (Stift et al., 2008). Alternatively, completely homolog chromosome tetrads arising from an autotetraploidization event may experience a secondary differentiation in two homolog pairs (Stift et al., 2008). This transition toward ploidy or diploidization is expected in polyploid species until a species may be considered to be functionally diploid. Even in fully diploidized species, residual evidence of polyploid ancestry (e.g. residual tetrasomy) is occasionally observed because of gene homology products (Allendorf and Thorgaard, 1984).

This is also evident in sturgeons where at least three polyploidization events occurred during their genome evolution (reviewed by Vasil'ev, 2009). On the other hand, Ludwig et al. (2001) proposed that not only genome polyploidization but also genome functional reduction took place in the evolution of sturgeon species. The functional genome reductions probably went through the differentiation of duplicated chromosome sets into distinct duplex, tetraplex and hexaplex of homeologs. Therefore a fraction of the sturgeon genome is still present in the tetraploid, octaploid and dodekaploid scale. This hypothesis is corroborated with the results of our study and is supported by different types of inheritance of different microsatellite alleles across sturgeon species, where disomic, tetrasomic and even octasomic inheritance have been observed by many authors (e.g. Ludwig et al., 2001; Rodzen and May, 2002; Rodzen et al., 2004; Fopp-Bayat, 2008 and others).

Another interesting and novel finding from this study was the occurrence of functional hexaploidy in *A. brevirostrum*. Blacklidge and Bidwell (1993) proposed that *A. brevirostrum* is an allopolyploid species, a descendant of ancestral spontaneous triploids (12n = 360 chromosomes). Fontana et al. (2008a) determined by means of fluorescent *in situ* hybridization (FISH) six fluorescent signals in analysed karyotypes, and they subsequently concluded a functional hexaploidy for this species. Therefore, given the evidence discussed, we conclude that *A. brevirostrum* should be classified as a functional hexaploid.

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CHAPTER 3

FIRST EVIDENCE OF AUTOTRIPLOIDIZATION IN STERLET (*ACIPENSER RUTHENUS*)

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ANIMAL GENETICS • ORIGINAL PAPER

First evidence of autotriploidization in sterlet (*Acipenser ruthenus*)

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Abstract Polyploidization has played an important role in vertebrate evolution. Acipenseridae bring clear examples of polyploidy ancestry and, also, polyploidization seems to be an ongoing process in these fishes. In the present study, the genetic origin of six triploid specimens morphologically determined as *Acipenser ruthenus* from commercial aquaculture was analyzed using a combination of mitochondrial and nuclear markers. A further five successive statistical analyses including median joining of mitochondrial DNA control region sequences, principal coordinate analysis (PCA), factorial correspondence analysis (FCA), STRUCTURE assignation, and NewHybrids status determination for microsatellite data were applied for the clarification of the origin of one extra chromosome set added in these triploids genomes. Although interspecific hybridization had been suggested as a source of these triploids, the statistical analyses showed that the investigated triploids originate from autotriploidization rather than from interspecific hybridization. Therefore, we conclude that a combination of molecular markers with suitable statistical analyses should be used to verify the origin of unusual ploidy level. Evidently, such an approach is critically essential in aquaculture, where interspecific hybridization is very common and usually detected by changes in ploidy levels only.

Keywords Acipenseriformes · Interspecific hybridization · Molecular markers - polyploidization · Triploids

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Introduction

Sturgeons are thought to be one of the oldest group of fishes, which have evolved more than 250 million years ago (Bemis et al. 1997). Their evolution is inherently connected with polyploidization events, which resulted in different ploidy levels of the present sturgeon species. Acipenseriformes species can be divided into three groups according to their number of chromosomes in cell nuclei: group A, with~120 chromosomes; group B, with~250–270 chromosomes (Blacklidge and Bidwell 1993); and group C, consisting of a single species, the shortnose sturgeon, *Acipenser brevirostrum*, with a chromosome number of around 360 (Fontana et al. 2008). Although the chromosome numbers of Acipenseriformes are more or less clear, their polyploidy status often remains unresolved, with conflicting opinions. Recent investigations suggest a recent or evolutionary scale of Acipenseriformes ploidy levels (reviewed by Vasil'ev 2009).

Nowadays, sturgeon and paddlefish populations are at a historically low level and their survival density depends heavily on stocking (Pikitch et al. 2005). Several reasons for sturgeon population decline could be found, including overharvesting, poaching, river damming (Birstein et al. 1997), and loss of genetic integrity through the interspecific hybridization (e.g., Ludwig et al. 2002, 2009). Nevertheless, sensible management, together with restocking, can

significantly support wild sturgeon populations, as was demonstrated in North America (Pikitch et al. 2005), but the origin of hatchery juveniles and maintaining the genetic integrity and purity of farmed stocks should be seriously considered.

Sturgeon aquaculture has been rapidly developing during the last 30 years, and the tetraploid Siberian sturgeon (*A. baerii*) became the most preferred species in European aquaculture (Bronzi et al. 1999). Furthermore, interspecific hybridization between diploid and tetraploid sturgeon species is very common in sturgeon aquaculture (Nikolyukin 1964; Birstein et al. 1997; Ludwig et al. 2009; Flajšhans and Vajcová 2000; and others) and usually results in hybrids with intermediate ~180 chromosome number (Gorshkova et al. 1996), which, in terms of the recent scale of ploidy levels, exhibit triploidy.

In the present study, we used a combination of nuclear and mitochondrial markers in conjunction with several statistical techniques to discriminate the origin of six sturgeon triploids observed in aquaculture and morphologically determined as *A. ruthenus*.

Materials and methods

Samples collection

Fin clips were collected from 96 specimens, including 24 samples of each sturgeon species commonly bred in European aquaculture (*A. ruthenus*, *H. huso*, *A. baerii*, and *A. gueldenstaedtii*). Simultaneously, six samples were taken from expected triploid specimens that were previously detected by means of flow cytometry during the routine screening of 110 *A. ruthenus* individuals. All samples originated from aquaculture farms in the Czech Republic (Rybníkářství Pohořelice a.s., the hatchery in Pohořelice; Rybářství Třeboň a.s., the hatchery in Mydlovary; and the hatchery of the Faculty of Fisheries and Protection of Waters, University of South Bohemia, České Budějovice, situated in Vodňany).

Flow cytometry

Peripheral blood was collected from the caudal vessel into a heparinized syringe according to Pravda and Svobodová (2003), kept at 4 °C, and samples were processed immediately by means of flow cytometry. With flow cytometry (Partec CCA I; Partec GmbH, Münster, Germany), a ploidy level of each specimen was verified as the relative DNA content in blood cells using 4′,6-diamidino-2-phenylindole (DAPI) according to Linhart et al. (2006). Erythrocytes of functionally diploid *A. ruthenus* gave the relative DNA content as the diploid standards.

DNA extraction, PCR amplification, sequencing, and fragmentation analysis

Genomic DNA was extracted from fin clips using the NucleoSpin® Tissue Kit (MACHEREY-NAGEL). Amplification followed a standard polymerase chain reaction (PCR) protocol to amplify a 505-bp mtDNA control region (Mugue et al. 2008). The PCR reaction was carried out in 30 μl under the following conditions: 95 °C for 120 s, 5 cycles at 95 °C for 60 s, 53 °C for 60 s, 72 °C for 60 s, followed by 30 cycles at 95 °C for 30 s, 53 °C for 45 s, 72 °C for 60 s, and a final extension at 72 °C for 12 min. The PCR products were purified using the NucleoSpin® Extract II Kit (MACHEREY-NAGEL) and sequenced in both directions using the professional sequencing service of Macrogen (Seoul, Korea).

Microsatellite DNA fingerprinting of seven microsatellite loci, Afu34, Afu39, Afu68 (May et al. 1997), Spl101, Spl163 Spl173 (McQuown et al. 2000), and Aox45 (King et al. 2001) were used. The PCR reaction was carried out in 10 μl according to a reaction profile described by Ludwig et al. (2009). The resulting fragment sizes were determined by the use of a Beckman Coulter CEQ 2000 automatic sequencer.

Statistical analysis

Mitochondrial DNA (mtDNA) control region sequences were aligned using MAFFT version 6.814b (Katoh et al. 2002), implemented in Geneious Pro 5.6.3. (Drummond et al. 2011; Biomatters Ltd., Auckland, New Zealand). The haplotype network was constructed using the median joining algorithm in Network version 4.6.1.0 (Bandelt et al. 1999). A principal coordinate analysis (PCA) was carried out by the use of GenAlEx version 6.4 (Peakall and Smouse 2006) to visualize the genetic relationship from the genetic distances matrix. A factorial correspondence analysis (FCA) was done with GENETIX software version 4.05 (Belkhir et al. 2004) in order to investigate the relationships among individuals. Assignment tests were performed with STRUCTURE software version 2.1 (Pritchard et al. 2000) and, finally, NewHybrids software (Anderson and Thompson 2002) was used for multidimensional analysis and the assignment test of assumed 3n specimens together with the analyzed species. The determination of the exact genotype is difficult for polyploid species and, furthermore, there is no statistical method that permits the analysis of polyploid data together with the diploid data. Because the aim of this study is hybrid identification, there was no determination of the "true" genotypes for polyploid patterns and no quantifications were achieved in this way.

Results

The data obtained from flow cytometry, mtDNA and microsatellite markers were analyzed to determine the origin of triploid specimens from aquaculture farms with respect to common interspecific hybridization in farmed sturgeon stocks on the one hand or possible spontaneous triploidization of pure sturgeon species on the other.

Ploidy level analysis

Flow cytometry revealed the erythrocyte DNA content of five *A. ruthenus* standards to be diploid. All the analyzed *A. ruthenus* and *H. huso* specimens were diploid, except for six *A. ruthenus* specimens, which were suggested to be triploids. All differences were found to be significant. Similarly, the erythrocyte DNA content of *A. gueldenstaedtii* and *A. baerii* specimens under study was found to be tetraploid. The coefficient of variation (C_V) in the erythrocyte DNA content was below or equal to 2.5 % for all specimens.

Structure of mtDNA haplotypes of the analyzed specimens

All triploid specimens shared haplotypes with diploid *A. ruthenus* (Fig. 1). All *A. ruthenus* haplotypes were significantly separated from *A. baerii* and *gueldenstaedtii* haplotypes (more than 100 polymorphic sites). As well as mtDNA haplotypes, differentiation between *A. ruthenus* and *H. huso* were supported by more than 60 polymorphic sites.

Genetic distances and PCA

In the first step, genetic distances were calculated and visualized by PCA (Fig. 2). The PCA displayed significant differences between the analyzed species. Axis 1 explained 35.5 % of the total genetic variation, while axis 2 explained 22.7 % of the total genetic variation. Four main clusters representing each sturgeon species were identified. Triploid specimens clustered with *A. ruthenus*, except for triploid specimen R185A placed between *A. ruthenus* and tetraploid species *A. baerii* and *A. gueldenstaedtii*. This might be considered as an indication that this individual could possibly be of hybrid origin.

Multidimensional analysis (FCA)

Subsequently, multidimensional analysis (FCA) was used to considered triploid specimens to be of hybrid origin or not (Fig. 3). The most informative was axis 1, which explained 38.7 % of the total genetic variation, while axes 2 and 3 represented 33.5 % and 27.8 % of the total genetic variation, respectively. Similarly to PCA, four main clusters were determined, corresponding to one of the analyzed sturgeon species. In contrast with PCA, all triploid specimens clustered together with *A. ruthenus*, where none of them were suggested to be of hybrid origin.

Assignment test

Four specific clusters were assigned by STRUCTURE for K from 2 to 5. Each cluster corresponded to one of the analyzed sturgeon species (Fig. 4). The pure specimens were strongly assigned in their corresponding species and all triploid specimens were associated with the *A. ruthenus* cluster. The Q values for the pure specimens in the ten independent runs were similar and the mean Q value for the pure species cluster was greater than 0.95 (Fig. 4)

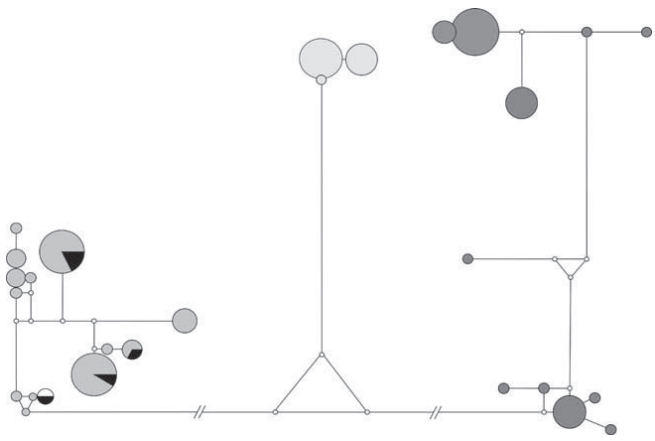

Fig. 1 Haplotype network constructed from the mtDNA control region partial sequence (505 bp) of the analyzed species: (●) 3n individuals; (◍) *A. ruthenus*; (○) *H. huso*; (◉) *A. baerii*; (●) *A. gueldenstaedtii*

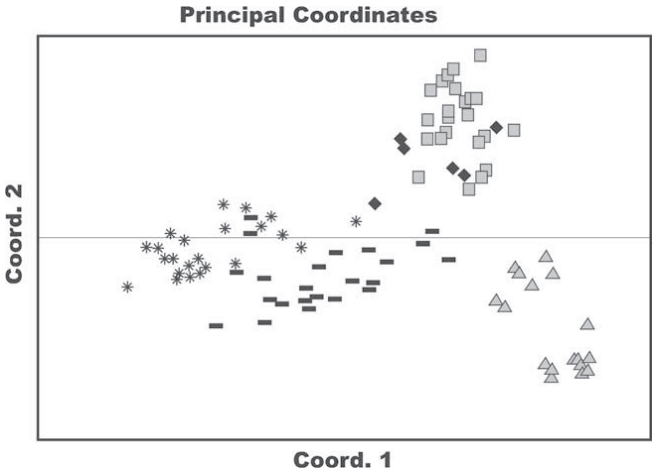

Fig. 2 Principal component analysis (PCA) based on seven microsatellite loci in four different sturgeon species and triploid individuals: (*diamonds*) 3n individuals; (*squares*) *A. ruthenus*; (*triangles*) *H. huso*; (*rectangles*) *A. baerii*; (*asterisks*) *A. gueldenstaedtii*

Determination of possible hybrids

The NewHybrids software was used to distinguish possible hybrid categories and hybrid origin of triploid specimens. All triploid specimens showed a probability higher than 0.99 to be composed of *A. ruthenus* only. None of them were assigned to be a hybrid with *H. huso*, *A. baerii*, or *A. gueldenstaedtii*. Backcross origin of these specimens was significantly excluded (probability lower than 0.005). The posterior probabilities for each individual are shown in Supplementary Table 1.

Discussion

The purpose of this study was to investigate the origin of six triploid specimens morphologically determined as *A. ruthenus* from aquaculture farms. Our results, based on the combination of nuclear and mitochondrial markers in conjunction with the analysis of several statistics, revealed that the investigated triploids originated by autotriploidization. These results give the first evidence of autotriploidization in Acipenseriformes given by molecular data.

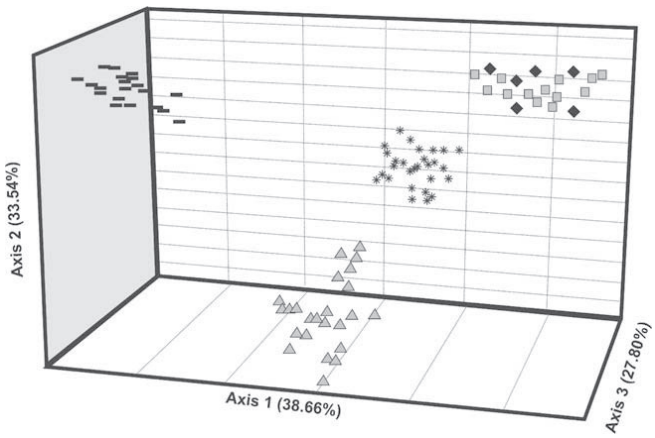

Fig. 3 Factorial correspondence analysis (FCA) based on seven microsatellite loci in four sturgeon species and triploid individuals: (*diamonds*) 3n individuals; (*squares*) *A. ruthenus*; (*triangles*) *H. huso*; (*rectangles*) *A. baerii*; (*asterisks*) *A. gueldenstaedtii*

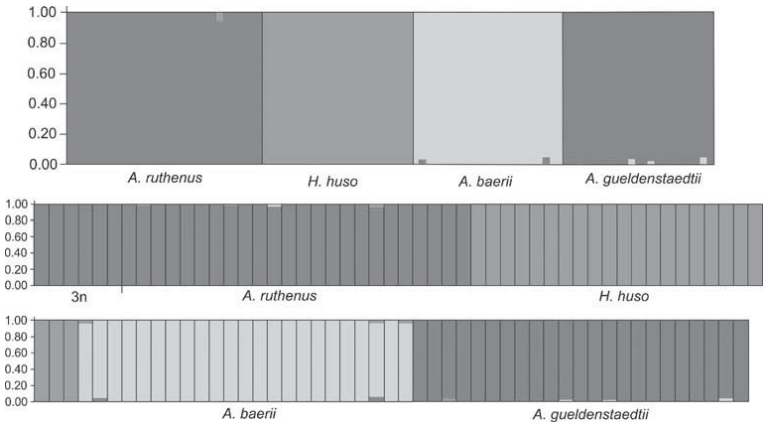

Fig. 4 Assignation of 99 sturgeons by STRUCTURE analysis based on seven microsatellite loci

Sturgeon triploids have been observed in wild populations (e.g., Ludwig et al. 2009; Dudu et al. 2011) and in aquaculture farms (e.g., Flajšhans and Vajcová 2000) several times, but interspecific hybridization between diploid and tetraploid sturgeon species has been considered to be the most probable origin of these triploids.

In addition, triploids can arise not only by interspecific hybridization but also by autotriploidization or dispermic fertilization (Vasil'ev 2009). Autopolyploidization played a major role in the evolution of salmonids (Allendorf and Thorgaard 1984) and catostomids (Ferris and Whitt 1980). Further spontaneous diploidization of the maternal chromosome set is a well-known event in fish and natural autotriploids, and has been described several times, e.g., in Characiformes (Morelli et al. 1983; Venere and Galetti 1985; Giuliano-Caetano and Bertollo 1990; Fauaz et al. 1994; Centofante et al. 2001; Malacrida 2002), Gymnotiformes (De Almeida Toledo et al. 1985), and Siluriformes (Borin et al. 2002). In these fish taxa, the fertilization of a non-reduced egg (2n) by a haploid sperm (1n) has been considered to be the most probable origin of triploidy. In the case of sturgeons, Fopp-Bayat (2007) described the occurrence of spontaneous normal diploid among gynogenetic haploids in *A. baerii* and, similarly, Fopp-Bayat and Woznicki (2007) observed spontaneous diploids among gynogenetic haploids in *A. ruthenus*. The presence of triploid individuals was also detected by Omoto et al. (2005) in normally fertilized eggs of the bester (hybrid between *H. huso* and *A. ruthenus*). Finally, Zhou et al. (2011) described the occurrence of triploid individuals among progeny obtained from the artificial propagation of *A. mikadoi*. These recent findings confirmed that spontaneous egg diploidization (usually by the junction of pronuclei in haploid egg as a result of suppressing the second meiotic division) might also be possible for Acipenseriformes species. In contrast, Vasil'ev (2009), in agreement with Van Eenennaam et al. (1996), concluded that the frequencies of spontaneous egg diploidization were close to zero in Acipenseridae.

Polyspermic fertilization is theoretically more probable in the case of sturgeons compared to other fish taxa, due to the presence of multiple micropyles in the eggs (Dettlaff et al. 1993). Nevertheless, Pšenicka et al. (2010) concluded that the creation of a cytoplasmic projection in the sturgeon egg after fusion with the spermatozoon blocks polyspermy in all other micropyles. This fact makes dispermic fertilization as a formation mechanism of sturgeon triploids improbable.

It is evident that autotriploids are not affected by an introgressive gene flow from "alien species" because they have added one extra chromosome set from the genome of the same species; however, their effect on the genetic integrity and purity of the diploid population remains unresolved.

The identification of sturgeon triploid origin without knowledge of their parental genotypes is still very complicated. Flow cytometry is able to detect triploids very quickly in large populations, but without any evidence about their origin. Similarly, karyotypes did not provide clear evidence on this issue because species-specific chromosome probes have not yet been published. Nuclear markers such as microsatellites are a useful tool for triploids identification, especially in combination with mtDNA analyses (e.g., Ludwig et al. 2009). However, using microsatellites, the origin of one extra chromosome set in the triploid genome cannot be discriminated due to the absence of diagnostic alleles or diagnostic loci among closely related sturgeon species (Ludwig 2008). On the other hand, Dudu et al. (2011) demonstrated that microsatellites in combination with suitable statistic analyses could be useful for hybrid

origin identification. Based on these findings, we supposed that similar procedures could be used for the discrimination of triploids origin of the sturgeons under study.

Analyses of mtDNA reflect only hybridization on the maternal lineage and, moreover, its analyses showed a limitation for the discrimination of closely related sturgeon species complex (e.g., *A. gueldenstaedtii*, *A. baerii*, *A. persicus*, and *A. naccarii*), due to the overlapping of mtDNA profiles (Birstein et al. 2000). A close relationship among analyzed *A. gueldenstaedtii* and *A. baerii* individuals was also evident from the results given in our study. Furthermore, higher intraspecific divergence within *A. gueldenstaedtii* under study was observed when compared with the other analyzed species (see Fig. 1). This finding was based on the very essence of the historic dispersal of *A. gueldenstaedtii* and isolation of its local populations as a result of geological and hydrological rearrangements of Eurasian water bodies, which had an effect on its genetic structure (Timoshkina et al. 2009). These changes have been actively introduced into the aquaculture population of *A. gueldenstaedtii* via artificial reproduction and intense fisheries (Bronzi et al. 1999).

Conclusion

The analysis of the mitochondrial DNA (mtDNA) control region did not reveal any evidence regarding the possible interspecific hybridization between diploid and tetraploid sturgeon species on maternal position because any tetraploid sturgeon haplotype has not been observed in the genomes of the investigated triploid specimens. Although principal component analysis (PCA) indicated the presence of one possible hybrid individual, multidimensional analyses (factorial correspondence analysis, FCA) and assignment tests have not confirmed this observation. Finally, the determination of possible hybrids using the NewHybrids software assigned all triploid specimens to be pure *Acipenser ruthenus* species, and none of them was assigned to be of hybrid or backcross origin.

Based on these findings, we concluded that the investigated triploids originated by autotriploidization. On the other hand, we were not able to clarify the mechanism of this autotriploidization due to the absence of parental individuals of these triploids.

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CHAPTER 4

FERTILITY OF A SPONTANEOUS TRIPLOID MALE SIBERIAN STURGEON (*ACIPENSER BAERII*)

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FERTILITY OF A SPONTANEOUS TRIPLOID MALE SIBERIAN STURGEON (*ACIPENSER BAERII*)

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ABSTRACT

Evolution of sturgeons and paddlefishes (order Acipenseriformes) is inherently connected with polyploidization events which resulted in differentiation of ploidy levels and chromosome numbers of present acipenseriform species. Moreover, allopolypoidization as well as autopolyploidization seems to be an ongoing process in these fishes and individuals with abnormal ploidy levels were occasionally observed within sturgeon populations. Here, we reported occurrence of Siberian sturgeon (*Acipenser baerii*) male with abnormal ploidy level for these species, accessed its ploidy level and chromosome number and investigate its potential sterility or fertility. The ploidy level of this specimen as well as hatchery stock of sterlet (*A. ruthenus*) Russian sturgeon (*A. gueldenstaedtii*) and Siberian sturgeon (*A. baerii*) was determined using flow cytometry, image cytometry of erythrocyte nuclei, karyotyping, and analysis of seven diagnostic microsatellite loci. Later on fish were stimulated with hormone injections and mutually cross-bred.

Acipenser ruthenus possessed 120 chromosomes, exhibiting recent diploidy (2n), *A. gueldenstaedtii* and *A. baerii* had ~ 245 chromosomes representing recent tetraploidy (4n), and *A. baerii* male with abnormal ploidy level had ~ 368 chromosomes, indicating recent hexaploidy (6n) which referred to its triploid origin. Genealogy assessed from the *mtDNA control region* did not reveal genome markers of other sturgeon species and this individual was supposed to be spontaneous triploid. Following hormone stimulation, the spontaneous triploid male produced the normal volume of sperm for *A. baerii* males, but spermatozoa concentration was significantly lower (0.10×10^9 ml^{-1} *vs.* $0.63 \pm 0.69 \times 10^9$ ml^{-1}). Fertilization of *A. baerii* and *A. gueldenstaedtii* ova from normal 4n level females with sperm of the spontaneous triploid male produced viable, non-malformed pentaploid (5n) progeny with a ploidy level intermediate to those of the parents.

This study firstly describes occurrence of triploid individual of *A. baerii* and confirmed its autopolyploid origin. In addition to that, the first detailed evidence about fertility of triploid sturgeon was provided in contrast to the general belief in sterility or subfertility of triploid fishes due to interference of gametogenesis.

Key Words: *Acipenseridae; polyploidy determination; sperm quality; triploid fertility; cross-breeding ploidy*

INTRODUCTION

Evolution of vertebrate genomes was possibly associated with three episodes of whole-genome duplication. The first occurred at the origin of vertebrates and another at the origin of gnathostomes, the 2R hypothesis (McLysaght et al., 2002; Panapoulou and Poustka, 2005). The third, 3R, is suggested to have occurred in fin-rayed teleostean fishes after their divergence from the earliest lineage of actinopterygians, sturgeon and paddlefish of the extant order Acipenseriformes (Ventakhesh, 2003; Hoegg et al., 2004; Volff, 2005; Froschauer et al., 2006).

Sturgeon, paddlefish, the fishes of the genera *Psephurus*, *Polyodon* (Acipenseriformes: Polyodontidae), *Acipenser, Huso, Scaphirhynchus*, and *Pseudoscaphirhynchus* (Acipenseriformes: Acipenseridae) provide the most remarkable examples of evolution of ploidy levels among vertebrates (Nelson, 2006). Independent of the two ancient genome duplication rounds, several lineage-specific duplication events occurred in sturgeons and paddlefishes (Ludwig et al., 2001; Fontana et al, 2007).

Within the most recent vertebrates, sturgeon is among the species with a large number of chromosomes. Nowadays, several well divided groups of acipenseriform species can be recognized depending on DNA content and the number of chromosomes in their cell nuclei. They include species with ~ 120, ~ 240 and ~ 360 chromosomes, corresponding to elevated DNA content (Birstein et al., 1993). Despite this widely respected division, ploidy status of Acipenseriformes often remains unresolved with conflicting opinions. Recent investigations suggest two scales of ploidy levels in Acipenseriformes: the 'evolutionary scale', which presumes tetraploid – octaploid – dodecaploid relationships among species, and the 'recent scale', which presumes diploid – tetraploid – hexaploid relationships.

Probably due to the polyploid nature of their genomes, sturgeon of differing ploidy levels hybridize both in nature (e.g. Birstein et al., 1997; Ludwig et al., 2002, 2009) and in captivity (Nikolyukin, 1964; Flajšhans and Vajcová, 2000 and others), suggesting mutual fertility of various sturgeon species irrespective of ploidy level. Some authors (Arefjev, 1997; Birstein, 2002 and Vasil´ev, 2009) assume hybrids of parent species having different ploidy levels to be sterile. The same generally applies to fertility of fishes with an odd number of chromosomes, such as autotriploids (Piferrer et al., 2009) in which meiosis is seriously affected because three homologous chromosomes cannot pair during the zygotene stage of prophase I, interfering with gonad development and gametogenesis. However, this phenomenon was considered species- and case-specific, since i) in some fish species autotriploids have been found to produce small amounts of aneuploid spermatozoa incapable of generating viable offspring if used for fertilization; ii) in several other species, spermatozoa of autotriploid males can induce egg activation leading to non-viable aneuploid embryos; and iii) in rare cases viable larvae have developed from normal or aneuploid eggs from a triploid female fertilized with sperm from triploid males (Piferrer et al., 2009).

In the present study, we report the occurrence of a spontaneous triploid male among hatchery stock of Siberian sturgeon, *Acipenser baerii*, a species of recent tetraploid level 4n with ~ 240 chromosomes; its experimental hybridization with normal females of *A. baerii* and *A. gueldenstaedtii*; and analysis of resulting viable

progeny. The main aims of this study were to accessed chromosome number and ploidy level of analyzed individuals and investigate potential sterility or fertility of observed spontaneous triploid male of *A. baerii.*

MATERIAL AND METHODS

This study was carried out in strict accordance with the Czech Law n. 246/1992 about "Animal welfare". Authors possess a testimony according to §17 of Law 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). Moreover, this was specifically approved by University of South Bohemia animal care committee. All surgery was performed under the clove oil anesthesia, and all efforts were made to minimize suffering.

Fish and breeding details

Parental fish originated from the hatchery of the Faculty of Fisheries and Protection of Waters, University of South Bohemia, one 15-year-old *A. baerii* male (1.19 m total length (L_T), 9.30 kg body mass (M_B)), suspected of being triploid after cytometric examination; one 14-year-old female *A. baerii* (1.15 m L_T, 9.50 kg M_B); and one 14-year-old *A. gueldenstaedtii* (1.40 m L_T, 13.60 kg M_B) were used for the experiment. Fifteen-year-old male *A. baerii* and *A. gueldenstaedtii* (1.05 m L_T and 8.25 kg M_B; 0.90m L_T and 7.00 kg M_B, respectively) were used for controlled breeding with both females. Five male sterlet, *Acipenser ruthenus*, (0.51 ± 0.15 m L_T; 0.65 ± 0.10 kg M_B) were used as the diploid standard for flow cytometry and karyotyping. Fish were kept in 5 m^3 indoor tanks supplied with re-circulating water at 14 °C for 7 days prior to hormone stimulation. Before handling, fish were anesthetized by immersion in 0.07 $ml.l^{-1}$ clove oil (Kolářová et al., 2007).

Males were stimulated with an intramuscular injection of 4 $mg.kg^{-1}$ M_B carp pituitary suspension in physiological saline 36 h before expected sperm sampling (Gela et al., 2008). Females were stimulated with an intramuscular injection of 0.5 $mg.kg^{-1}$ M_B carp pituitary suspension in physiological saline 42 h before expected ovulation, and again 12 h later with 4.5 $mg.kg^{-1}$ M_B of the same suspension (Gela et al., 2008). Ovulated eggs for the cross-breeding experiments were collected after microsurgical incision of oviducts as described by Štěch et al. (1999). Fin clips were collected from all parent fish and stored in 96% ethanol.

Sperm quantity and quality

Sperm was collected from the seminal duct, using a 5 mm diameter plastic cannula, into a 100 ml tissue culture flask following the protocol of Gela et al. (2008). Samples were maintained on crushed ice at 0 to 4 °C. Sperm volume and spermatozoa concentration, motility, and velocity were assessed according to Linhart et al. (2000). Spermatozoa viability (% live) was determined by epifluorescence microscopy of dual-stained sperm DNA (Flajšhans et al., 2004). Wherever applicable, samples were processed in triplicate.

Fertilization trials

Eggs of *A. baerii* and *A. gueldenstaedtii* were inseminated with sperm from the supposed triploid *A. baerii*. Pure-breeding of *A. baerii* and *A. gueldenstaedtii* males and females was conducted as control. Eggs were put into plastic beakers in 50 g aliquots, which were placed on a shaking table with constant 200 rpm and 10 mm deflection. Each aliquot was inseminated with 1.5 ml of sperm and activated with 200 ml dechlorinated tap water at 15.0 °C. After 2 min, fertilized eggs from each aliquot were separately distributed into 200 cm^3 incubator cages, supplied with UV sterilized re-circulating tap water at 15.0 °C, 9 mg l^{-1} O_2 in triplicate. During incubation, eggs and hatched larvae were counted, and dead eggs were counted and removed. Hatching rate was computed as described by Linhart et al. (2006). Embryos, swimming-up larvae, and/or early juveniles were sampled for analysis.

Ploidy level analyses

Peripheral blood was collected from the caudal vessel into a heparinized syringe (Pravda and Svobodová, 2003), kept at 4 °C, and processed immediately with flow cytometry (Partec CCA I; Partec GmbH, Münster, Germany) for karyotyping of leucocyte cultures. The ploidy of each adult fish was measured as relative DNA content in blood cells, using 4´,6-diamidino-2-phenylindole (DAPI) first separately for erythrocytes and spermatozoa, and then pooled (Linhart et al., 2006). Erythrocytes and spermatozoa of a functionally diploid *A. ruthenus* gave relative DNA content of 2n as the diploid and 1n as the haploid standard. Thirty samples of swimming-up larvae from each fertilization trial were processed for flow cytometry (Lecommandeur et al., 1994).

For image cytometry, slides were conventionally stained with Giemsa and inspected microscopically (Olympus BHS microscope NCSPlanApo 60x dry objective coupled to a 3CCD Sony DXC-9100P color camera). At least 100 erythrocyte nuclei per specimen were recorded and analyzed by Olympus MicroImage v. 4.0 software. Erythrocyte nuclear area (NA, μm^2) was assessed following the protocol in Flajšhans (1997). The NA of functionally diploid *A. ruthenus* provided the diploid standard.

For karyotyping, metaphase chromosomes were prepared from leucocytes of peripheral blood (Fujiwara et al., 2001). Blood was collected in a heparinized syringe (Zentiva) from the caudal vein and the syringe left in an upright position at 4 °C overnight. The sedimented leucocytes and erythrocytes were cultured separately in complete medium (T 199 – Sigma, FBS Superior – Baria, Antibiotic Antimycotic Solution – Sigma, Kanamycin monosulfate – Sigma, LPS – Sigma, PHA H15 – Biomedica, Mercaptoethanol – Sigma) for 6 days at 20 °C. The cell suspension was prepared routinely by harvesting cells after colchicine treatment, hypotonization, and fixation. A drop of the cell suspension was placed on a microscope slide, dried, and stained with 5% Giemsa stain buffered to pH 7.0. The chromosomes from embryos and early juveniles were prepared according to Völker and Kulmann (2006).Metaphase chromosome plates were examined microscopically (Olympus AX 70) and recorded with an Olympus DP30VW digital camera. Karyotypes were arranged using Ikaros MetaSystems (Metasystems, Germany) software.

Molecular analyses of mitochondrial DNA

The genomic DNA was extracted from fin-clips using the NucleoSpin®tissue kit (MACHEREY-NAGEL). Amplification followed a standard PCR protocol to amplify a 620 bp mtDNA fragment of the control region (Mugue et al., 2008). The PCR reaction was carried out under the following conditions: 95 °C for 120 s, 5 cycles of 95 °C for 60 s, 53 °C for 60 s, and 72 °C for 60 s; 30 cycles of 95 °C for 30 s, 53 °C for 45 s, and 72 °C for 60 s; and a final extension at 72 °C for 12 min. The PCR products were purified using NucleoSpin® Extract II (MACHEREY-NAGEL) and sequenced in both directions by Macrogen (Seoul, Korea). Sequences were aligned using Geneious 5.4 software (Drummond et al., 2011) and BLASTed against the NCBI nucleotide collection using Mega-BLAST. This database contains previously published sequences of the control region for most sturgeon species (http://www.ncbi.nim.nih.gov/).

Paternity analysis

The genomic DNA from 24 swimming-up larvae fixed in 96% ethanol from each trial, as well as from fin-clips of all parent fish was extracted using the NucleoSpin®tissue kit (MACHEREY-NAGEL). Microsatellite DNA fingerprinting of seven microsatellite loci, Afu19, Afu34, Afu39, Afu68 (May et al., 1997), Spl101, Spl173 (McQuown et al., 2000), and Aox45 (King et al., 2001), was used. PCR were performed on a volume of 25 µl, containing 1 U Taq DNA polymerase, 10 pmol of each primer, 10–50 ng DNA, 100 µM of each dNTP, 2.5 mm $MgCl_2$, and 2.5 µl 10 x incubation buffer. Amplifications were performed under the following conditions: one cycle at 94 °C for 3min and 25 cycles at 94 °C followed by locus-specific annealing conditions: 53 °C for loci Aox 45, AfuG 54, and AfuG 135; 55 °C for locus Afu 54; 57 °C for loci Afu 34, Afu 68, Spl 101, Spl 163, Spl 173, and Atr 114; and 59 °C for locus Spl 105; followed by 72 °C for 30s and a final extension at 72 °C for 10min. The PCR products were inspected on agarose gel, then run in the ABI 3110 DNA analyzer. Genotypes were scored using GeneMapper v4.1 (Applied Biosystems, TM).

Statistics

The association of ploidy with the studied variables was assessed by ANOVA followed by Tukey multiple-range test in Statistica 9.1 software (StatSoft, Inc., 2010). Values of $P < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Confirmation of ploidy levels of analyzed individuals

Flow cytometry revealed the erythrocyte DNA content of the 5 males of *A. ruthenus* standard to be diploid (2n; Fig. 1, peak 1), 1 male and 1 female *A. baerii* to be tetraploid (Fig 1, peak 2) and one specimen of *A. baerii* to be 1.38-fold that of the tetraploids (Fig. 1, peak 3). Sperm of analyzed males had average DNA content equivalent to

haploidy (1.0n; Fig. 2, peak 1), diploidy (2.0n; Fig. 2, peak 2), and triploidy (3.0n; Fig. 2, peak 3), respectively. The erythrocyte DNA content of the *A. gueldenstaedtii* male and female under study was also tetraploid. The coefficient of variation (C_V) in both erythrocyte and sperm DNA content was below or equal to 2.5% for all specimens. The relative DNA content in swimming-up larvae from the normal x spontaneous triploid *A. baerii* and normal *A. gueldenstaedtii* x spontaneous triploid *A. baerii* hybridizations (Fig. 3a) and from pure-breeding *A. baerii* and *A gueldenstaedtii* (Fig. 3b) revealed 100% intermediate ploidy level of the F_1 hybrid larvae compared to 100% normal ploidy in the purebred larvae.

Image cytometry analysis revealed mean erythrocyte nuclear area for *A. ruthenus* (19.39 ± 1.43^a μm²) confirming diploidy, *A. baerii* (29.95 ± 1.30^b μm²) and *A. gueldenstaedtii* (30.1 ± 1.05^b μm²) confirming tetraploidy and one specimen of *A. baerii* (39.59 ± 3.56^c μm²) to be 1.32-fold that of the tetraploids. These results correspond to already published data (Bytyutskyy et al., 2012).

Chromosome analysis of individuals under study revealed *A. ruthenus* specimens with chromosome number 2n = 120, *A. baerii* and *A. gueldenstadtii* with modal chromosome number 2n ~ 245, while the *A. baerii* specimen with higher ploidy level exhibited chromosome number 2n ~ 368. Except for microchromosomes, all chromosomes of *A. ruthenus* could be paired (Fig. 4a). Those of *A. baerii* grouped in quadruplets (Fig. 4b), and those of the *A. baerii* specimens with higher ploidy level grouped into hexaplets (Fig. 4d), indicating spontaneous triploidization.

Karyotype analysis of specimens of the normal x spontaneous triploid *A. baerii* hybrid revealed modal number 2n ~ 300 chromosomes, with macrochromosomes that grouped into apparent pentaplets (Fig. 4c). The same ploidy level of progeny and karyotype structure resulted from crossing of normal *A. gueldenstadtii* female x spontaneous triploid *A. baerii* male.

Ploidy levels of *A. baerii* and *A. gueldenstadtii* females used for cross-breeding corresponded to ~ 245 chromosomes as previously reported for these species (Fontana et al., 2001). Chromosome number 2n = 120 of *A. ruthenus* used as ploidy standard also corresponded to findings of a number of other cytogenetic studies (Ráb et al., 1996; Fontana et al., 1999). Ploidy level of progeny resulting from crossing *A. baerii* and *A. gueldenstaedtii* with the spontaneous triploid *A. baerii* male was intermediate between the ploidy levels of parents at ~ 300 chromosomes. This observation was in accordance with findings of Gorshkova et al. (1996) and in contrast to the report of Arefjev et al. (1991) who demonstrated non-intermediate karyotypes in progeny of hybridization of beluga, *Huso huso*, and *A. gueldenstaedtii*. The control mating of normal ploidy level *A. baerii* and *A. gueldenstaedtii* yielded progeny of the same ploidy level as the parents. Our results clearly demonstrated that sturgeon with different ploidy levels readily interbreed, as assumed for various combinations of sturgeon species (Birstein et al., 1997).

Confirmation of origin of the triploid male *A. baerii*

The nucleotide sequence of the *A. baerii* spontaneous triploid male was closely similar (99%, E = 0.0) to *A. baerii* haplotype FJ843094.1 (Wang et al., 2009). A maximum of three variable sites were detected in common with *A. baerii* sequences (Ludwig et al., 2009). Similar results were obtained for the *A. baerii* female. The *A.*

gueldenstaedtii female had mtDNA haplotype closely similar (97%, E = 0.0) to *A. gueldenstaedtii* haplotype FJ843096 (Wang et al., 2009).

Sturgeon triploids have been observed in wild populations (Ludwig et al., 2009; Dudu et al., 2011) and in aquaculture (Flajšhans and Vajcová, 2000), and interspecific hybridization has been considered the most probable origin of these triploids. Triploids can also arise through autotriploidization mechanisms such as spontaneous diploidization of the maternal chromosome set (SDM) (Cherfas et al., 1995; Aegerter and Jalabert, 2004; Ezaz et al., 2004) or by dispermic fertilization (Vasiľev, 2009). The SDM is a well-known event in fish, and natural autotriploids resulting from fusion of a haploid sperm pronucleus with an SDM oocyte have been described (Cherfas et al., 1995; Centofante et al., 2001; Borin et al., 2002; Aegerter and Jalabert, 2004; Ezaz et al., 2004).

Because there is no sturgeon species with a higher ploidy level and chromosome number than *A. brevirostrum* (6n ~ 372 chromosomes; Fontana et al., 2008), the hybrid origin of the *A. baerii* triploid might seem improbable. Bearing in mind that a triploid of an evolutionary tetraploid species is a hexaploid, the origin of the extra two chromosome sets in the genome of the observed specimen might be derived from autotriploidization, specifically by the junction of pronuclei in a haploid egg, as a result of suppressing the second meiotic division, i.e. by the SDM mechanism. This statement is supported by findings of triploid individuals among progeny obtained from artificial propagation of *A. mikadoi* (Zhou et al., 2011). Furthermore, these triploids showed a model chromosome number of 360-402 in somatic cells (Zhou et al., 2013), similarly to triploid described in our study. The occurrence of triploid individuals has also been detected in normal fertilized eggs of the bester (hybrid of *H. huso and A. ruthenus*) and SDM caused by egg over-ripening was suggested as the most probable cause (Omoto et al., 2005). Finally, spontaneous autopolyploids of *A. transmontanus* have also been recently reported (Drauch Schreier et al., 2011). These findings are contradictory to conclusions of Vasiľev [18] who stated that occurrence of spontaneous egg diploidization is close to zero in acipenserids.

Confirmation of fertility of the triploid male *A. baerii* via sperm characteristics

Data on sperm volume per 1 kg M_B, numbers of spermatozoa in 1 ml sperm, viability of spermatozoa and their motility and velocity are given in Table 1. The mean velocity of spermatozoa 15 s after activation was the only variable significantly associated with ploidy level in *A. baerii* (169.66 ± 8.69 vs. 152.04 ± 15.53 $\mu m.s^{-1}$ for the normal and spontaneous triploid individual, respectively). Velocity of spermatozoa of *A. ruthenus* was 159 ± 19.25 $\mu m.s^{-1}$, similar to the spontaneous triploid *A. baerii*. Mean percent of motile spermatozoa did not differ between these males. Sperm viability assay revealed similar rates of live spermatozoa (98.76 ± 0.36%, 95.93 ± 2.58%, and 93.47 ± 8.51% for the *A. ruthenus*, normal *A. baerii*, and spontaneous triploid *A. baerii* males, respectively).

Triploids are commonly supposed to be infertile or subfertile; nevertheless triploid fish occasionally produce a small amount of sperm (Piferrer et al., 2009). The observed *A. baerii* triploid produced the same volume of sperm as the diploid specimens. All

sperm parameters observed in this experiment were within the range reported by other authors. Spermatozoa concentration in sturgeon is generally reported in the range of 1–4 x 10^9 ml^{-1} (Dettlaff and Ginzburg, 1993; Hochleitner, 1996). Reported sperm concentration of *A. ruthenus* from aquaculture is 0.25–1.5 x 10^9 ml^{-1} (Dzyuba et al., 2012) and is 1.68–2.42 x 10^9 ml^{-1} for *A. baerii* (Piros et al., 2002). High rates of spermatozoa motility indicated high quality of sperm and corresponded with high viability. Spermatozoa velocity of the triploid *A. baerii* was lower than reported for males of normal ploidy level, as also observed by Pšenička et al. (2011) in studies of the same species. These authors considered the higher relative DNA content in spermatozoa of the triploid *A. baerii* to be the cause of the diference.

Confirmation of fertility of the triploid male *A. baerii* via analyses of its progeny

Experimental fertilization of ova of the normal *A. baerii* and *A. gueldenstaedtii* female with sperm of the spontaneous triploid *A. baerii* resulted in hatching rates of 64.16 ± 2.60% and 36.64 ± 2.06%, respectively, similar to that of *A. baerii* and *A. gueldenstaedtii* pure-breeding (50.56 ± 8.19% and 39.82 ± 3.64%). No apparent increase in the percent of malformed non-viable larvae was observed in either cross-breeding experiment.

Parental genotypes and observed genotypes of progeny at informative loci (*Aox45*) from experimental crossbreeding (Table 2) confirmed Mendelian segregation patterns in all specimens analyzed. The inheritance of maternal alleles from the normal female *A. gueldenstaedtii* and spontaneous triploid male *A. baerii* were clearly evident at two highly informative loci, *Aox45* and *Afu68*. The observed genotypes were predominately composed of two maternal and three paternal alleles. The genotypes observed are listed in electronic supplementary material.

Fertilization of ova of *A. baerii* and *A. gueldenstaedtii* with normal ploidy with sperm of the spontaneous triploid *A. baerii* produced fully viable progeny with ploidy level intermediate to those of the parents. This demonstrated full fertility of the spontaneous triploid male, contrary to the general belief in sterility or subfertility of triploid fish due to interference with gametogenesis. In order to better understand fertility in triploid fish, it is necessary to distinguish between allo- and auto-triploidy, as well as between the effects of triploidy in females and males (Piferrer et al., 2009). Allotriploid fish can arise through multiplication of chromosome sets resulting from natural intergeneric or interspecific hybridization, which sometimes give rise to altered gametogenetic mechanisms and reproductive modes resulting in polyploidy and clonal or hemiclonal inheritance (Ráb et al., 2006). Such natural diploid-polyploid hybrid complexes and cryptic species complexes have been described, especially in Eurasian cyprinid genera *Cobitis* and *Carassius*, in the Iberian minnow *Squalius alburnoides*, Oriental weatherfish *Misgurnus anguillicaudatus*, and in stone loach *Barbatula barbatula* exhibiting fertile allotriploid females and/or males (Piferrer et al., 2009). These authors point out that autotriploids arise through multiplication of chromosome sets within a species, which can occur due to disruption of gametogenesis caused by cytogenetic alterations of meiosis, to suppression of the second meiotic division due to cytoskeletal alterations in post-ovulatory age oocytes, or to disruption of gamete fertilization, e.g. by polyspermy. Meiosis in autotriploids is seriously affected, as three homologous chromosomes cannot correctly pair during

the zygotene stage of prophase I (Carrasco et al., 1998; Cuñado et al., 2002). In females, these meiotic disruptions affect follicular assemblage and oocyte growth (Benfey, 1999), and, as a result, autotriploid females compared to the diploid exhibit rudimentary ovaries or ovarian tissue with fewer, immature, oocytes (Benfey, 1999; Felip et al., 2001; Flajšhans et al., 2010). On the other hand, meiosis in males takes place with the onset of puberty after spermatogonia have been through many cycles of cell division by mitosis, and testes of triploid males can develop similar to diploids, with functional steroidogenic cells (Benfey et al., 1986; Felip et al., 2001; Linhart et al., 2006; Piferrer et al., 2009). In some marine and freshwater fish species, autotriploid males produce viable aneuploid spermatozoa incapable of egg fertilization (Benfey and Sutterlin, 1984; Benfey et al., 1986; Piferrer et al., 1994; Linhart et al., 2006), while in case of autotriploid tench, *Tinca tinca*, the fertilization potential of its sperm has been described (Hulák et al., 2010).

Meiosis in the triploid male *A. baerii* under study may not have been affected by these factors, because triploidy in evolutionary tetraploid *A. baerii* resulted in hexaploidy. The homologous chromosomes could correctly group in triplets during the zygotene stage of prophase I during spermatogenesis. The findings of this study contradict the statement that autohexaploid specimens originating from autotriploidization will be sterile, as are artificially produced autotriploid fishes (Vasiľev, 2009).

CONCLUSION

We confirmed the spontaneous triploid status of the examined *A. baerii* male, i.e. corresponding to ~ 368 chromosomes of the somatic cells and haploid sperm, a characteristic which has not been reported in other sturgeon species so far.

This study demonstrated that a triploid sturgeon male may be fertile owing to its polyploid ancestry and release viable and motile spermatozoa, and that these might have full fertilization potential resulting in a hatching rate similar to that of purebred sturgeon and produce viable progeny with ploidy levels intermediate between those of the parents. Based on these findings, it might be assumed that (auto)triploids of other sturgeon species could be fertile as well.

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Table 1. *Relative volume of sperm kg^{-1} body mass (M_B), concentration and viability of spermatozoa (spz) and motility and velocity 15 s post activation (mean ± SD) in sperm of A. ruthenus, A. gueldenstaedtii, A. baerii and of the triploid 6n A. baerii. Values with the same superscript letter do not differ significantly at P < 0.05.*

Males	No. of Fish	Relative Volume of Sperm (ml kg^{-1} bm)	Concentration (109 ml^{-1})	Viability (% live spz)	Motility (% motile spz)	Velocity (µm s^{-1})
A. ruthenus	6	13.57 ± 4.69^{a}	1.75 ± 0.46^{a}	98.76 ± 0.36^{a}	100.00 ± 12.10^{a}	159.00 ± 19.25^{ab}
A. gueldenstaedtii	6	8.6 ± 4.6	1.54±0.80	92.56 ± 3.2	90.60±9.20	165.20±1572
A. baerii	6	22.14 ± 7.07^{a}	1.52 ± 1.88^{a}	95.93 ± 2.58^{a}	95.24 ± 9.85^{a}	169.66 ± 8.69^{b}
A. baerii triploid	1	24.29^{a}	2.35 ± 0.05^{a}	93.47 ± 8.51^{a}	100.00 ± 5.22^{a}	152.04 ± 15.53^{a}

Table 2. *Fragmentation analyses results at locus Aox45 for hybridization of A. gueldenstaedtii female and A. baerii triploid male.*

Sample	Marker	Allele 1	Allele 2	Allele 3	Allele 4	Allele 5	Allele 6	Allele 7	Allele 8	Allele 9
A. gueldenstaedtii female	Aox45	121				142	145			157
A. baerii triploid male	Aox45		124	127	136	142		148	151	
progeny 1	Aox45	121	124				145	148	151	
progeny 2	Aox45	121	124		136	142			151	
progeny 3	Aox45				136	142		148		157
progeny 4	Aox45			127		142		148		157
progeny 5	Aox45		124			142			151	157
progeny 6	Aox45	121	124	127			145		151	
progeny 7	Aox45	121	124				145	148	151	
progeny 8	Aox45		124				145	148	151	157
progeny 9	Aox45			127		142		148		157
progeny 10	Aox45		124	127		142	145			157
progeny 11	Aox45	121	124	127	136					157
progeny 12	Aox45	121	124			142			151	157
progeny 13	Aox45		124	127		142			151	157
progeny 14	Aox45	121	124		136	142			151	
progeny 15	Aox45		124		136	142				
progeny 16	Aox45	121	124		136	142		148		
progeny 17	Aox45	121		127		142	145		151	
progeny 18	Aox45			127		142	145	148		157
progeny 19	Aox45	121	124		136	142			151	
progeny 20	Aox45	121	124	127			145		151	
progeny 21	Aox45		124	127		142			151	157
progeny 22	Aox45			127		142		148		157

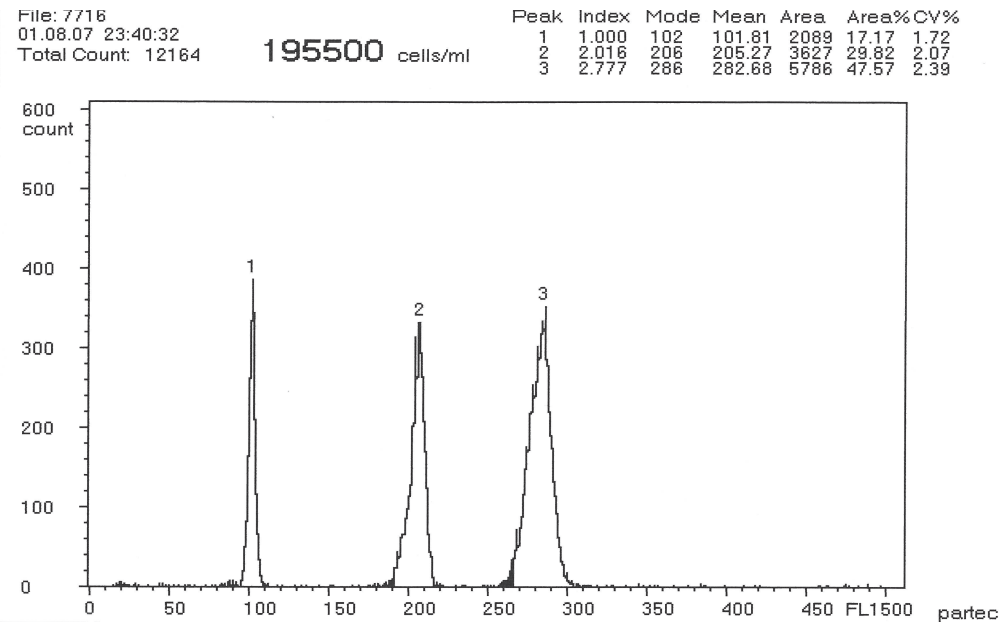


Figure 1. *Flow cytometric histogram showing relative DNA content in blood cells of sterlet (A. ruthenus; peak 1), Siberian sturgeon (A. baerii; peak 2) and spontaneous triploid Siberian sturgeon (A. baerii) (peak 3).*

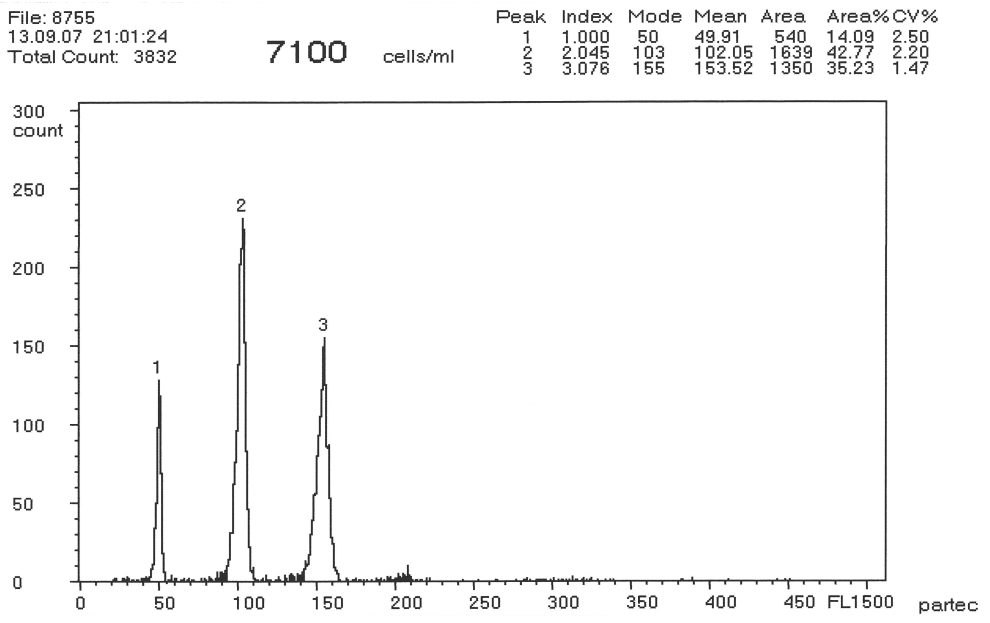


Figure 2. *Flow cytometric histogram showing relative DNA content in sperm of sterlet (A. ruthenus; peak 1), Siberian sturgeon (A. baerii; peak 2) and spontaneous triploid Siberian sturgeon (A. baerii) (peak 3).*

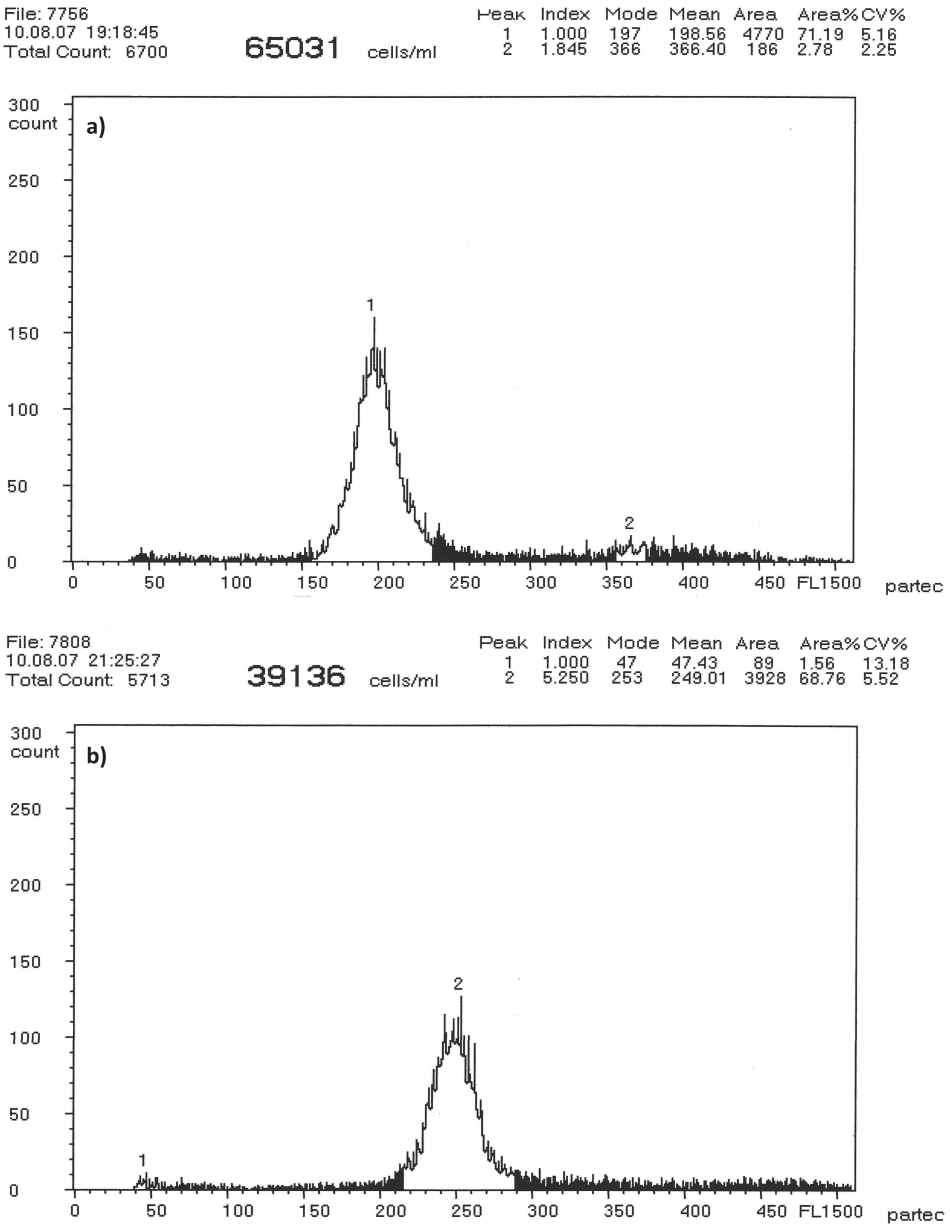
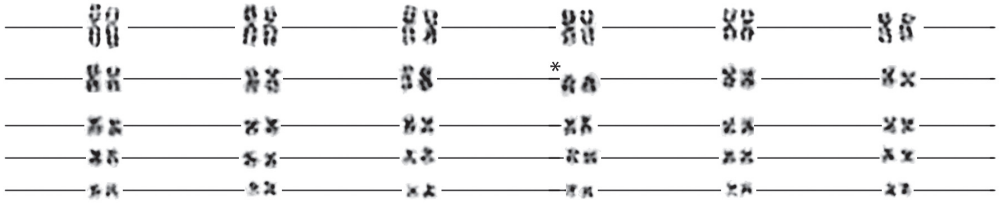
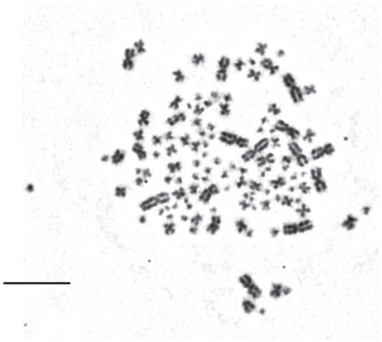


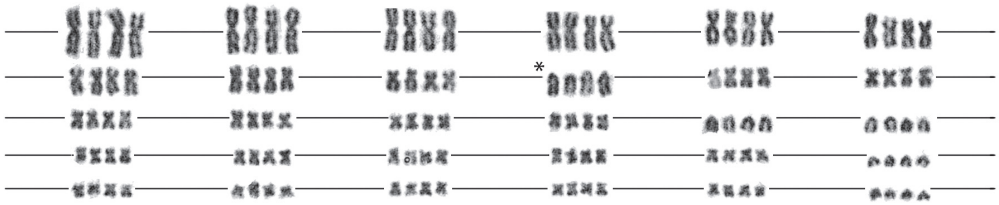
Figure 3. *Flow cytometric histogram showing relative DNA content in tissue suspension of swimming-up larvae from A. baerii and A gueldenstaedtii purebreeding (a) and hybridizations of the normal x spontaneous triploid A. baerii and the normal A. gueldenstaedtii x spontaneous triploid A. baerii (b).*

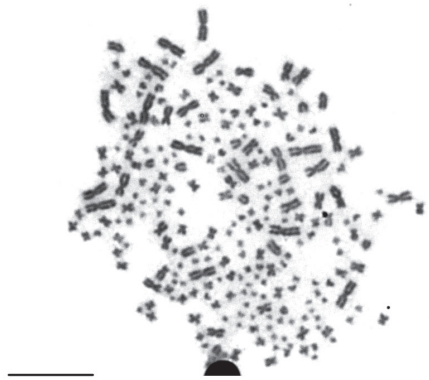
a





b





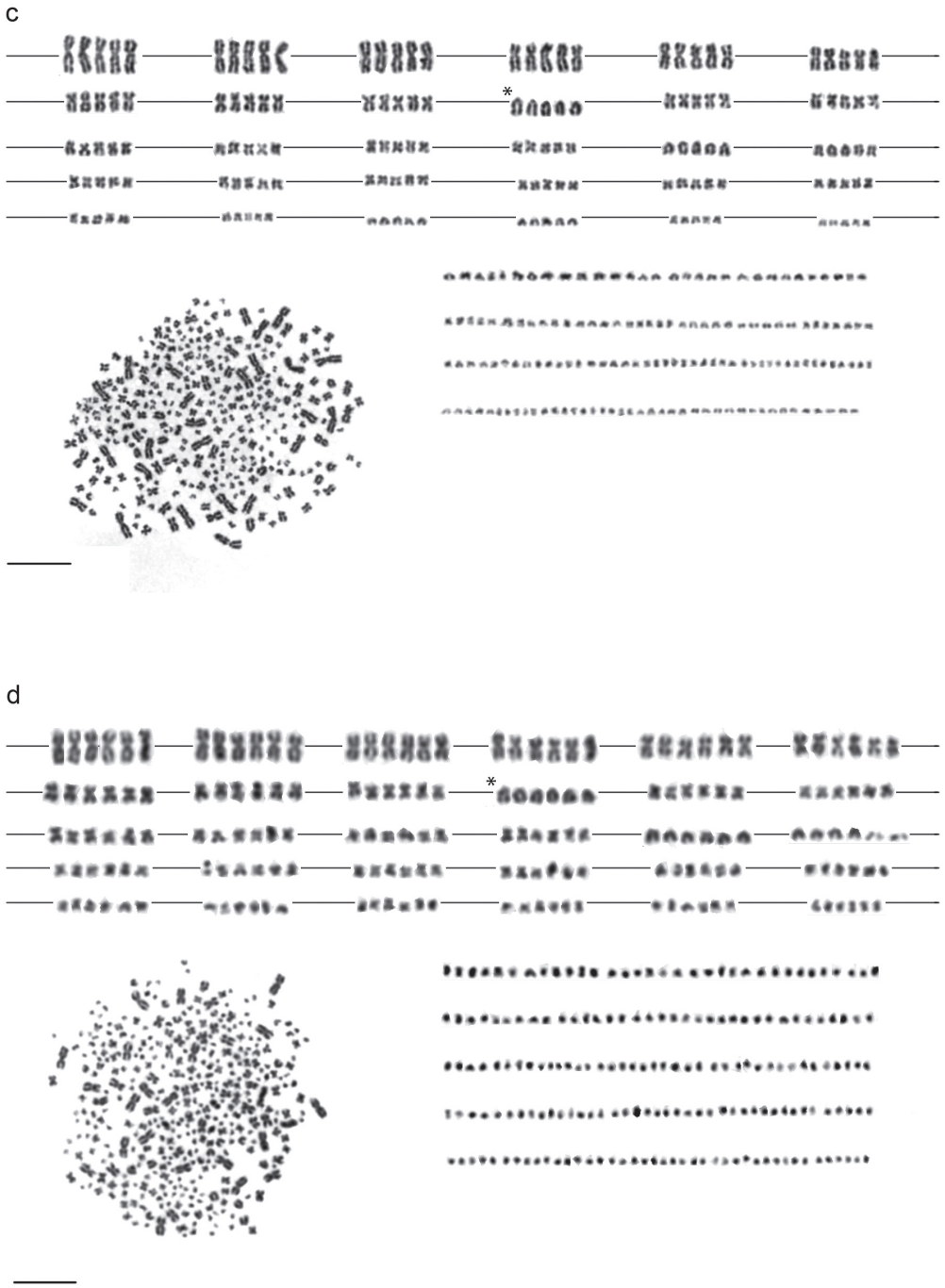


Figure 4. *Metaphase cell and corresponding karyotype arranged from Giemsa-stained chromosomes of (a) sterlet Acipenser ruthenus (2n = 120), (b) Siberian sturgeon A. baerii (2n ~ 240), (c) Siberian sturgeon A. baerii (hybrid of 2n ~ 240, x spontaneous triploid 2n ~ 360 resulting in 2n ~ 300) and (d) spontaneous triploid 2n ~ 360; asterisks denotes „acipenserine" cytotaxonomic marker. Bars equal 10 μm.*

CHAPTER 5

INVESTIGATION OF THE SEGREGATION PATTERN OF MICROSATELLITE ALLELES DURING EXPERIMENTAL HYBRIDIZATION OF POLYPLOID STURGEON SPECIES

Unpublished data

INVESTIGATION OF THE SEGREGATION PATTERN OF MICROSATELLITE ALLELES DURING EXPERIMENTAL HYBRIDIZATION OF POLYPLOID STURGEON SPECIES

The main aim of this study was to observe segregation pattern of microsatellite alleles in the course of hybridization of polyploid sturgeon species and also ploidy levels of obtained hybrid progeny.

MATERIAL AND METHODS

All parental fishes originated from the hatchery of the Faculty of Fisheries and Protection of Waters, University of South Bohemia and their ploidy levels were firstly investigated by flow cytometry (see following text). One functional tetraploid female of *A. gueldenstaedtii*, two functional tetraploid females of *A. baerii*, one diploid male of *A. ruthenus*, one functional tetraploid male of *A. baerii* and one functional hexaploid male of *A. baerii* were used for the following experimental crossbreeding. All fishes were hormonally stimulated and artificially reproduced according to procedure described in Chapter 4.

Eggs were inseminated according to the following matrix:

Type of crossbreeding	Female (ploidy levels)		Male (ploidy levels)		Expected ploidy levels of obtained progeny
1st	*A. baerii* (4n)	x	*A. ruthenus* (2n)	=	3n
2nd	*A. baerii* (4n)	x	*A. baerii* (4n)	=	4n
3rd	*A. baerii* (4n)	x	*A. baerii* (6n)	=	5n
1st	*A. gueldenstaedtii* (4n)	x	*A. ruthenus* (2n)	=	3n
2nd	*A. gueldenstaedtii* (4n)	x	*A. baerii* (4n)	=	4n
3rd	*A. gueldenstaedtii* (4n)	x	*A. baerii* (6n)	=	5n

Eggs were put into plastic beakers in 50 g aliquots, which were placed on a shaking table with constant 200 rpm and 10 mm deflection. Each aliquot was inseminated with 1.5 ml of sperm and activated with 200 ml dechlorinated tap water at 15.0 °C. After 2 min, fertilized eggs from each aliquot were separately distributed into 200 cm^3 incubator cages, supplied with UV sterilized re-circulating tap water at 15.0 °C, 9 $mg.l^{-1}$ O_2 in triplicate. Embryos, swimming-up larvae, and/or early juveniles were sampled for analyses.

Ploidy levels of all parental fishes were investigated by means of flow cytometry from peripheral blood collected from the caudal vessel (Linhart et al., 2006) while ploidy levels of swimming-up larvae from each fertilization trait were investigated also by flow cytometry but from tissue suspension (Lecommandeur et al., 1994). All ploidy levels were measured as relative DNA content using 4′,6-diamidino-2-phenylindole (DAPI) and erythrocytes of a functionally diploid *A. ruthenus* gave relative DNA content of 2n as the diploid standard.

For molecular analyses, genomic DNA from 22 swimming-up larvae fixed in 96% ethanol from each trial, as well as from fin-clips of all parent fish was extracted using the NucleoSpin®tissue kit (MACHEREY-NAGEL). Microsatellite DNA fingerprinting of seven microsatellite loci, Afu19, Afu34, Afu39, Afu68 (May et al., 1997), Spl101, Spl173 (McQuown et al., 2000), and Aox45 (King et al., 2001), was used. PCR were performed in same conditions as described in Chapter 4. The PCR products were inspected on agarose gel, then run in the ABI 3110 DNA analyzer. Genotypes were scored using GeneMapper v4.1 (Applied Biosystems, TM).

RESULTS

Flow cytometry revealed the erythrocyte DNA content of all pure parental species referred to their natural status with the expectation of one male specimen of *A. baerii* to had 1.38-fold of DNA content if compared to tetraploids of the same pure species. The relative DNA content of swimming–up larvae obtained from hybridization of parental species having different ploidy levels revealed 100% intermediate ploidy to those of parental species, while pure crossbreeding of *A. baerii* and hybridization between *A. baerii* female and *A. gueldenstaedtii* male provided progeny with the same ploidy as was borne by their parents.

The observed genotypes at informative loci confirmed Mendelian segregation patterns in all analyzed specimens. The observed genotypes of progeny were predominately composed of one allele from diploid specimen and two alleles from tetraploid specimen in case of the first type of crossbreeding, while the genotypes obtained from the second type of crossbreeding were composed of four alleles (two alleles inherited from each of the parental species. Finally, progeny resulting from the third type of crossbreeding had two alleles from tetraploid specimen and three from hexaploid one. The fragmentation results and genotypes at informative loci for each type of crossbreeding were displayed in the following tables (Tab. 1 through 6).

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Table 1. *Fragmentation analyses results at locus Spl 101 for hybridization of A. baerii tetraploid female with A. ruthenus diploid male (♀ – maternal alleles; ♂ – paternal alleles; ♀♂ – alleles inherited from both parents).*

Sample	Marker	Allele 1	Allele 2	Allele 3	Allele 4	Allele 5
A.baerii female	Spl101	220♀		240♀	248♀	256♀
A.ruthenus male	Spl101	220♂	236♂			
progeny 1	Spl101		236♂	240♀		256♀
progeny 2	Spl101	220♀	236♂	240♀		
progeny 3	Spl101		236♂	240♀	248♀	
progeny 4	Spl101	220♀	236♂		248♀	
progeny 5	Spl101	236♂		240♀	248♀	
progeny 6	Spl101		236♂		248♀	256♀
progeny 7	Spl101	220♀	236♂		248♀	
progeny 8	Spl101	220♀	236♂	240♀		
progeny 9	Spl101	220♀	236♂		248♀	
progeny 10	Spl101	220♀	236♂			256♀
progeny 11	Spl101	220♀♂		240♀		
progeny 12	Spl101	220♀	236♂		248♀	
progeny 13	Spl101	220♀	236♂	240♀		
progeny 14	Spl101	220♀♂		240♀		
progeny 15	Spl101	220♀	236♂	240♀		
progeny 16	Spl101	220♀♂		240♀		
progeny 17	Spl101	220♀	236♂		248♀	
progeny 18	Spl101	220♀	236♂	240♀		
progeny 19	Spl101	220♀♂				256♀
progeny 20	Spl101	220♀♂		240♀		
progeny 21	Spl101		236♂	240♀		256♀
progeny 22	Spl101	220♂		240♀		256♀

Table 2. *Fragmentation analyses results at locus Spl 173 for hybridization of A. gueldenstaedtii tetraploid female with A. ruthenus diploid male (♀ – maternal alleles; ♂ – paternal alleles).*

Sample	Marker	Allele 1	Allele 2	Allele 3	Allele 4	Allele 5	Allele 6
A.gueldenstaedtii female	Spl173		228♀	232♀		240♀	248♀
A.ruthenus male	Spl173	220♂			236♂		
progeny 1	Spl173	220♂	228♀			240♀	
progeny 2	Spl173	220♂	228♀				248♀
progeny 3	Spl173	220♂				240♀	248♀
progeny 4	Spl173		228♀		236♂	240♀	
progeny 5	Spl173		228♀	232♀	236♂		
progeny 6	Spl173	220♂		232♀			248♀
progeny 7	Spl173		228♀	232♀			
progeny 8	Spl173			232♀		240♀	
progeny 9	Spl173		228♀		236♂	240♀	
progeny 10	Spl173	220♂	228♀	232♀			
progeny 11	Spl173	220♂		232♀			248♀
progeny 12	Spl173	220♂				240♀	248♀
progeny 13	Spl173						
progeny 14	Spl173			232♀	236♂	240♀	
progeny 15	Spl173		228♀		236♂	240♀	
progeny 16	Spl173		228♀		236♂	240♀	
progeny 17	Spl173		228♀		236♂	240♀	
progeny 18	Spl173		228♀	232♀	236♂		
progeny 19	Spl173		228♀		236♂		248♀
progeny 20	Spl173	220♂	228♀			240♀	
progeny 21	Spl173		228♀	232♀	236♂		
progeny 22	Spl173	220♂	228♀			240♀	

Table 3. *Fragmentation analyses results at locus Spl 173 for purebreeding of tetraploid A. baerii (♀ – maternal alleles; ♂ – paternal alleles; ♂♂ – if two same alleles were inherited from paternal specimen).*

Sample	Marker	Allele 1	Allele 2	Allele 3	Allele 4	Allele 5	Allele 6
A.baerii female	Spl173	220♀	240♀		248♀		256♀
A.baerii male	Spl173			244♂		252♂	
progeny 1	Spl173				248♀	252♂♂	256♀
progeny 2	Spl173			244♂	248♀	252♂	256♀
progeny 3	Spl173	220♀			248♀	252♂♂	
progeny 4	Spl173		240♀	244♂		252♂	256♀
progeny 5	Spl173			244♂	248♀	252♂	256♀
progeny 6	Spl173			244♂	248♀	252♂	256♀
progeny 7	Spl173		240♀			252♂♂	256♀
progeny 8	Spl173		240♀	244♂		252♂	256♀
progeny 9	Spl173	220♀		244♂		252♂	256♀
progeny 10	Spl173	220♀	240♀	244♂		252♂	
progeny 11	Spl173		240♀		248♀	252♂♂	
progeny 12	Spl173	220♀				252♂♂	256♀
progeny 13	Spl173	220♀	240♀			252♂♂	
progeny 14	Spl173	220♀		244♂		252♂	256♀
progeny 15	Spl173		240♀	244♂	248♀	252♂	
progeny 16	Spl173				248♀	252♂♂	256♀
progeny 17	Spl173		240♀	244♂		252♂	256♀
progeny 18	Spl173		240♀			252♂♂	256♀
progeny 19	Spl173	220♀	240♀	244♂		252♂	
progeny 20	Spl173	220♀				252♂♂	256♀
progeny 21	Spl173	220♀	240♀	244♂		252♂	
progeny 22	Spl173	220♀				252♂♂	256♀

Table 4. *Fragmentation analyses results at locus Aox 45 for hybridization of A. gueldenstaedtii tetraploid female with A. baerii tetraploid male (♀ – maternal alleles; ♂ – paternal alleles).*

Sample	Marker	Allele 1	Allele 2	Allele 3	Allele 4	Allele 5	Allele 6	Allele 7	Allele 8
A.gueldenstaedtii female	Aox45	121♀				142♀	145♀		157♀
A.baerii male	Aox45		124♂	130♂	139♂			151♂	
progeny 1	Aox45		124♂				145♀	151♂	157♀
progeny 2	Aox45			130♂	139♂	142♀	145♀		
progeny 3	Aox45			130♂	139♂	142♀	145♀		
progeny 4	Aox45	121♀	124♂			142♀		151♂	
progeny 5	Aox45	121♀	124♂				145♀	151♂	
progeny 6	Aox45	121♀			139♂		145♀	151♂	
progeny 7	Aox45		124♂			142♀		151♂	157♀
progeny 8	Aox45		124♂			142♀		151♂	157♀
progeny 9	Aox45	121♀	124♂			142♀		151♂	
progeny 10	Aox45				139♂	142♀		151♂	157♀
progeny 11	Aox45	121♀	124♂					151♂	157♀
progeny 12	Aox45			130♂	139♂		145♀		157♀
progeny 13	Aox45			130♂		142♀		151♂	157♀
progeny 14	Aox45	121♀	124♂					151♂	157♀
progeny 15	Aox45			130♂	139♂	142♀	145♀		
progeny 16	Aox45	121♀	124♂				145♀	151♂	
progeny 17	Aox45	121♀		130♂	139♂		145♀		
progeny 18	Aox45	121♀			139♂		145♀	151♂	
progeny 19	Aox45	121♀		130♂	139♂		145♀		
progeny 20	Aox45	121♀	124♂	130♂					157♀
progeny 21	Aox45	121♀		130♂			145♀	151♂	
progeny 22	Aox45	121♀	124♂				145♀	151♂	

Table 5. *Fragmentation analyses results at locus Aox 45 for hybridization of A. baerii tetraploid female with A. baerii hexaploid male (♀ – maternal alleles; ♂ – paternal alleles; ♀♂ – alleles inherited from both parents; * origin of alleles was not possible to estimated).*

Sample	Marker	Allele 1	Allele 2	Allele 3	Allele 4	Allele 5	Allele 6	Allele 7
A.gueldenstaedtii female	Aox45		127♀	130♀			148♀	
A.baerii male	Aox45	124♂	127♂		136♂	142♂	148♂	151♂
progeny 1	Aox45	124♂		130♀		142♂	148♀	151♂
progeny 2	Aox45		127♀		136♂	142♂	148♀	151♂
progeny 3	Aox45	124♂		130♀		142♂	148♀	151♂
progeny 4	Aox45	124♂	127♀	130♀	136♂	142♂		
progeny 5	Aox45		127*	130♀		142♂	148*	
progeny 6	Aox45		127*		136♂	142♂	148*	
progeny 7	Aox45	124♂	127*	130♀	136♂		148*	
progeny 8	Aox45	124♂	127*				148*	151♂
progeny 9	Aox45		127*		136♂	142♂	148*	
progeny 10	Aox45		127♀♂			142♂	148♀♂	
progeny 11	Aox45		127*	130♀		142♂	148*	
progeny 12	Aox45	124♂		130♀	136♂		148*	
progeny 13	Aox45		127♀♂		136♂		148♀♂	
progeny 14	Aox45							
progeny 15	Aox45			130♀	136♂	142♂	148♀♂	
progeny 16	Aox45		127♀♂		136♂		148♀♂	
progeny 17	Aox45		127*	130♀	136♂		148*	
progeny 18	Aox45		127*	130♀	136♂		148*	
progeny 19	Aox45		127*	130♀	136♂		148*	
progeny 20	Aox45		127*			142♂	148*	151♂
progeny 21	Aox45	124♂	127*	130♀	136♂		148*	
progeny 22	Aox45	124♂		130♀			148*	151♂

Table 6. *Fragmentation analyses results at locus Aox 45 for hybridization of A.gueldenstaedtii tetraploid female with A. baerii hexaploid male (♀ – maternal alleles; ♂ – paternal alleles; ♀♂ – same alleles inherited from both parents; ♀♀♂ – two same maternal alleles and one paternal allele).*

Sample	Marker	Allele 1	Allele 2	Allele 3	Allele 4	Allele 5	Allele 6	Allele 7	Allele 8	Allele 9
A. gueldenstaedtii female	Aox45	121♀				142♀	145♀			157♀
A. baerii triploid male	Aox45		124♂	127♂	136♂	142♂		148♂	151♂	
progeny 1	Aox45	121♀	124♂				145♀	148♂	151♂	
progeny 2	Aox45	121♀	124♂		136♂	142♀			151♂	
progeny 3	Aox45				136♂	142♀		148♂		157♀
progeny 4	Aox45			127♂		142♀♂		148♂		157♀
progeny 5	Aox45		124♂			142♀♂			151♂	157♀
progeny 6	Aox45	121♀	124♂	127♂			145♀		151♂	
progeny 7	Aox45	121♀	124♂				145♀	148♂	151♂	
progeny 8	Aox45		124♂				145♀	148♂	151♂	157♀
progeny 9	Aox45			127♂		142♀♂		148♂		157♀
progeny 10	Aox45		124♂	127♂		142♂	145♀			157♀
progeny 11	Aox45	121♀	124♂	127♂	136♂					157♀
progeny 12	Aox45	121♀	124♂			142♂			151♂	157♀
progeny 13	Aox45		124♂	127♂		142♀			151♂	157♀
progeny 14	Aox45	121♀	124♂		136♂	142♀			151♂	
progeny 15	Aox45		124♂		136♂	142♀♀♂				
progeny 16	Aox45	121♀	124♂		136♂	142♀		148♂		
progeny 17	Aox45	121♀		127♂		142♂	145♀		151♂	
progeny 18	Aox45			127♂		142♂	145♀	148♂		157♀
progeny 19	Aox45	121♀	124♂		136♂	142♀			151♂	
progeny 20	Aox45	121♀	124♂	127♂			145♀		151♂	
progeny 21	Aox45		124♂	127♂		142♀			151♂	157♀
progeny 22	Aox45			127♂		142♀♂		148♂		157♀

CHAPTER 6

GENERAL DISCUSSION
ENGLISH SUMMARY
CZECH SUMMARY
ACKNOWLEDGEMENTS
LIST OF PUBLICATIONS
TRAINING AND SUPERVISION PLAN DURING STUDY
CURRICULUM VITAE

GENERAL DISCUSSION

Sturgeon ploidy levels

Large number of chromosomes and microchromosomes makes evaluation of ploidy level of acipenseriform species very complicated. This is moreover enhanced by long evolution of sturgeon genome where genome duplication and functional reductions events played very important role (Birstein et al., 1997; Ludwig et al., 2001, Peng et al., 2007). Ploidy relationships among extant groups of Acipenseriformes have been determined by applying a variety of techniques such as chromosome counts (e.g. Fontana, 2002), cell comparison and genome size estimation (e.g. Birstein et al., 1993; Blacklidge and Bidwell, 1993; Zhou et al., 2011; Bytyutskyy et al., 2012) and also by banding pattern of microsatellite markers (Ludwig et al., 2001). Despite of this wide range of techniques and increasing availability of modern molecular methods, ploidy levels of recent acipenseriform species remain unresolved in literature with many conflicting opinions.

Generally, results obtained from investigation of whole genome of sturgeon species usually supported evolutionary scale of their ploidy levels while results obtained from investigation of banding pattern of different molecular markers rather supported recent ploidy scale of sturgeon species. This might be caused by complicated evolutionary history of sturgeon species via genome duplication and functional reduction events.

In the first study (Havelka et al., 2013a; Chapter 2) ploidy levels of eleven sturgeon species from three ploidy groups were investigated using set of eleven highly polymorphic microsatellite markers. Microsatellite markers could permit direct although partial view into the sturgeon genome (Ludwig et al., 2001) and might be also useful tool for estimation of ploidy levels of almost all members of Acipenseriformes even though these markers were originally developed only for several sturgeon species as was reviewed in Chapter 1. This is allowed by the conservation of microsatellite flanking region among related species which suggest that developed markers for one species would be useful for family – wide scale as was already demonstrated also in sturgeons (e.g. May et al., 1997; McQuown et al., 2000, King et al., 2001, Ludwig et al., 2001, Jenneckens et al., 2001, Welsh et al., 2003 and others).

Maximum number of alleles observed at given locus provides evidence of ploidy level at that locus. Maximally two alleles, differing in size (diploid allelic band pattern) are expected for diploid locus, four different alleles (tetraploid allelic band pattern) for tetraploid locus and so on. However, estimation of true ploidy levels of acipenseriform species using microsatellites is more complicated due to their polyploid ancestry. The presence of two alleles at given locus may indicate diploidy, but cannot exclude tetraploidy and frequent genome duplication and functional reduction during sturgeon evolution might also misrepresent true ploidy levels of investigated species. Furthermore finding of maximal number of alleles within investigated individuals may be negatively influenced by low level of polymorphism which is typical problem in species with small population size such as in sturgeons and paddlefishes (Ludwig et al., 2001). It means that large panel of microsatellite markers

and sufficient numbers of analyzed individuals are necessary to use for accurate investigation of sturgeon ploidy levels, in order to avoid some misunderstandings.

In agreement with Ludwig et al. (2001) we classified species with ~ 120 chromosomes as functional (recent) diploids and species with ~ 240–270 chromosomes as functional (recent) tetraploids. This suggestion was based on observation of strict diploid allelic band pattern at seven investigated loci and tetraploid allelic band pattern at the same loci respectively. This classification of sturgeon ploidy levels was also supported by observation of Fontana (1994). This author observed localization of nucleolar organizer regions (NORs) on two morphologically different chromosomes in *A. ruthenus*, while in *A. baerii, A. transmontanus and A. naccarii* NORs were localized on eight chromosomes arranged in quadruplets. Moreover, observation of four duplicated loci in genomes of investigated species in our study (Havelka et al., 2013a; Chapter 2) referred to coexistence of diploid/tetraploid and/or tetraploid/octaploid allelic band patterns which might be ascribed to a secondary functional genome reduction as was assumed by Ludwig et al. (2001). While these authors observed only two duplicated loci and only in species with ~ 240–270 chromosomes we provided more detailed evidence by observation of four duplicated loci in all investigated species.

We generally agreed with common consideration that diploid ancestor of all Acipenseriformes had 60 chromosomes. Several linage specific duplication events must have occurred which resulted in present chromosome and ploidy diversity of sturgeon species (Birstein et al., 1997).

On the other hand, secondary functional genome reduction took also place in sturgeon evolution undoubtedly. These reductions went probably through the differentiation of duplicated chromosomes into the distinct duplex, tetraplex and hexaplex of homeologs. Because species with ~ 120 chromosomes are considered to be basal group of Acipenseriformes (Krieger et al., 2008), they had longer time for these reduction events which resulted in fact that functional genome reduction was nearly finished in this group (Ludwig et al., 2001). Nevertheless, presence of tetraploid allelic band patters in species with ~ 120 chromosomes and octaploid allelic band pattern in species with ~ 240–270 chromosomes at four loci investigated in our study (Havelka et al., 2013a; Chapter 2) showed that functional reduction of sturgeon genome is still an ongoing process and that some fractions of sturgeon genome still exhibit the tetraploid, octaploid and dodecaploid conformation. This situation has been already described even in fully diploidized species, in which residual evidence of polyploid ancestry (e.g. residual tetrasomy) were occasionally observed (Allendorf and Thorgaard, 1984).

Based of our results we would dare to offer solution of long problem of sturgeon ploidy scale classification. We considered that many conflicting opinions were caused by interpretation of data obtained by different methods without any respect to sturgeon genome evolution. As a result of genome duplication events, number of chromosomes, number of alleles at given locus, genome size and nuclear size have been multiplied (Birstein et al., 1993; Blacklidge and Bidwell, 1993; Ludwig et al., 2001; Fontana 2002; Zhou et al., 2011; Bytyutskyy et al., 2012 and many others). Nevertheless, functional genome reductions were carried out only at the molecular level but did not concern whole genomes. Later on, studies which investigated sturgeons' ploidy levels using analyses of molecular markers or chromosome probes usually referred to recent ploidy scale mainly because they affected just only molecular

nature of sturgeons' genome conformation. This was evident from data obtained in our study (Havelka et al., 2013a; Chapter 2) and also from other studies (e.g. Fontana, 1994; Ludwig et al., 2001; Fontana, 2002; Fontana et al., 2008). However, some of these studies also pointed to the evolutionary background of sturgeon ploidy levels. As an example of it could be seen observation of duplicated microsatellite loci Afu 57 and Afu 68 (May et al., 1997) in species with ~ 240–270 chromosomes by Ludwig et al. (2001) as well as four duplicated microsatellite loci observed across all analyzed species in our study (Havelka et al., 2013a; Chapter 2).

In contrast to the molecular markers, chromosome numbers and content of DNA in cell nuclei had not been reduced by functional reduction events and they still reflect evolutionary ploidy scale without any respect to recent sturgeon genome evolution via functional reductions. It might be the reason way almost all authors who used these techniques (e.g. Ohno et al., 1969; Birstein and Vasiľev, 1987; Birstein et al., 1993; Blacklidge and Bidwell, 1993; Birstein and DeSalle, 1998; Flajšhans and Vajcová, 2000) supported evolutionary ploidy scales of sturgeon species. Based on these considerations we concluded that there should not be any conflict in sturgeon ploidy levels but its interpretation must be strictly connected with detailed description of technique used for ploidy level estimation. Due to the fact that sturgeon species of the same ploidy level exhibit different nuclear DNA contents (Zhou et al., 2011; Bytyutskyy et al., 2012), we also highly recommend to discriminate terminologically between chromosomal ploidy and DNA ploidy of sturgeon species, similarly it has been suggested by Suda et al. (2006) for plants.

Some misunderstanding also occurred in discrimination of ploidy levels of some sturgeon species. In our study (Havelka et al., 2013a; Chapter 2) we brought novel evidence about hexaploid genome conformation of *A. brevirostrum* given by microsatellite data. Using the genome size estimation, Blacklidge and Bidwell (1993) supposed this species to be evolutionary dodecaploid (12n) and they suggested that *A. brevirostrum* originated from spontaneous triploidization of hybrids between evolutionary tetraploid and evolutionary octaploid sturgeons' species. In respect to observation of twelve alleles differing in size at locus Atr 114 (Rodzen and May 2002) in our study (Havelka et al., 2013a, Chapter 2) we generally approved that *A. brevirostrum* was correctly classified as evolutionary dodecaploid by Blacklidge and Bidwell (1993). On the other hand, based on finding of six alleles differing in size at seven investigated loci, we also agreed with Fontana et al. (2008), that this *A. brevirostrum* could be also classified as a functional (recent) hexaploid. Our observation was in contrast to Ludwig et al. (2001) who classified this species as functional (recent) octaploid based on observation of eight alleles differing in size at locus Afu 54 (May et al. 1997). Nevertheless, these authors correctly mentioned that low sample size (they were able to obtained only six samples due to the endangered status of *A. brevirosturm*) could cause misinterpretation of analyzed data.

Origin of abnormal ploidy levels of sturgeon species

As abnormal ploidy levels are usually called these, which are out of typical ploidy of pure sturgeon species. Nevertheless, expression "abnormal" sounds questionable in respect to the sturgeon evolution via genome duplication and functional reduction which is probably still an ongoing process. Nobody knows if exactly these individuals

with "abnormal" ploidy levels are not another step in complicated sturgeon evolution. Both way, these individuals were commonly observed in wild populations as well as in sturgeon aquaculture stocks and their origin might be explained by several ways.

Interspecific hybridization between species with different chromosome numbers and ploidy levels is widely accepted mechanism of formation of "abnormal" ploidy levels in sturgeons. Hybrids resulted from hybridization between species with different chromosome numbers and ploidy levels were considered to have chromosome number and ploidy level intermediated to those parental species (Gorshkova et al., 1996) while hybrids resulted from hybridization between different sturgeon species with the same chromosome number and ploidy level displayed ploidy and chromosome number as had been borne by their parents (Ene et al., 2001; Birstein, 2002). The changes in ploidy levels and chromosomes numbers had to be necessarily tied to the molecular level and their observation by microsatellite markers was possible as we demonstrated in our results (Chapter 5). By experimental crossing of recent diploid *A. ruthenus* with recent tetraploid *A. baerii* or *A. gueldenstaedtii* we obtained individuals with mainly triploid allelic band patters at analyzed loci in which two alleles were inherited from recent tetraploid specimen and one allele from recent diploid specimen. These exactly reflected basic principles of chromosome segregation during the meiosis of polyploid species (recently reviewed by Cifuentes et al., 2010). Similarly Ludwig et al. (2009) observed eight individuals with more then two alleles in one or more analyzed microsatellite loci in Upper Danube and based on observation of mtDNA haplotypes of *A. ruthenus* they identified five of these individuals as hybrids.

Another proof of formation of "abnormal" ploidy levels via interspecific hybridization of sturgeons was provided in Chapter 4. Experimental hybridization of fertile triploid *A. baerii* male with "normal" females of *A. baerii* and *A. gueldenstaedtii* were done and resulted progeny as well as parental species were analyzed using a wide scale of methods. Obtained results showed that analyzed progeny had intermediate DNA content, allelic band patters at analyzed microsatellite loci, cell nuclear area and chromosome number to those of the parents referring to recent pentaploidy. Bearing in mind that spontaneous triploid of recent tetraploid *A. baerii* was hexaploid (see Results in Chapter 4.) microsatellite locus Aox 45 (King et al., 2001) allowed estimation of pattern of gene segregation during described crossbreeding of this specimen. Obtained recent pentaploid progeny had three microsatellite alleles inherited from spontaneous triploid *A. baerii* male and two alleles inherited from "normal" recent tetraploid females.

In addition to that, the first detailed evidence about fertility of triploid sturgeon was provided by these results in contrast to the general belief in sterility or subfertility of triploid fishes due to interference of gametogenesis (Piferrer et al., 2009). In respect to these results, fertility of sturgeons with "abnormal" ploidy levels (possibly hybrids) must be seriously considered. If these hybrids with "abnormal" ploidy level were fertile, it could result in genetic contamination of genepools of pure sturgeon species which could cause irreparable loss of species genetic integrity. Fertility and later hybridization of specimens with "abnormal" ploidy levels might directly affect ploidy of resulted individuals as was described by Symonová et al. (2010) in detail. Using flow cytometry, image cytometry of erythrocyte nuclear dimensions and direct karyotyping, these authors described sturgeon individuals with evolutionary ploidy levels and chromosome numbers varied from

paleopentaploid 5n (~ 150 chromosomes), through paleohexaploid 6n (~ 180 chromosomes) and paleoheptaploid 7n (~ 210 chromosomes), to paleonanoploid 9n (~ 270 chromosomes) and paleodecaploid 10n (~ 300 chromosomes). These ploidy plasticity described by these authors originated from systematic experimental crossing of individuals with "abnormal" ploidy levels with pure *A. ruthenus, A. baerii* and *A. gueldenstaedtii* (Symonová et al., 2010). While even ploidy levels observed by Symonová et al. (2010) could be explained from molecular point of view without any problems, odd ploidy levels observed by these authors upon cytogenetic methods could not be expalained by molecular results due to the functional reduction events as mentioned in the previous text.

Differences in ploidy levels and chromosome numbers can be theoretically used for determination of sturgeon species. In adition to that, "abnormal" ploidy levels were considered as a marker for sturgeon hybrid detection (Arefjev, 1999; Ene et al., 2001), however in light of our recent findings (Havelka et al., 2013b; Chapter 3) is evident that this procedure could not be accepted as an only method for the hybrid detection.

Observed autotriploids of *A. ruthenus* in study Havelka et al. (2013b, Chapter 3) and also confirmation of non hybrid origin of spontaneous triploid of *A. baerii* described in Chapter 4. showed that autopolyploidization could be seen as another explanation for formation of "abnormal" ploidy levels in order Acipenseriformes. Generally, autopolyploidization is the process where chromosome sets are multiplied within one species (Piferrer et al., 2009). This process might occur by several mechanisms: i) dearrangement of gametogenesis caused by cytogenetic alternations on meiosis (Cherfas et al., 1995); ii) suppression of second mitotic division due to cytoskeletal changes in oocytes (Aegerter and Jalabert, 2004; Aegerter et al., 2005; Ezaz et al., 2004; Flajšhans et al., 2007); iii) disruption of the process of gamete fertilization (e.g. polyspermy; Grunina et al., 1995; Recoubratsky et al., 1996; Kirankumar and Pandian, 2004; Grunina et al., 2006).

In fish, autopolyploidization is not a rare event and it played important role in evolution of salmonids (Allendorf and Thorgaad, 1984) and castostomids (Ferris and Whitt, 1980). In sturgeon fishes, autopolyploidization is theoretically more probable compared to other fish taxa, due to the presence of multiple micropyles in the eggs which might be important prerequisite for possible polyspermic fertilization (Dettlaff et al., 1993). In such case, sturgeon egg would have to be fertilized by two or more spermatozoa as was described by Grunina et al. (2006). They produced dispermic androgenetic progeny of *A. stellatus* by dispermic fertilization of eggs by concentrated sperm. Similarly Grunina et al. (2011) produced androgenetic progeny of *A. baerii* with same protocol as in previous case, but using cryopreserved sperm. Nevertheless, Pšenička et al. (2010) suggested polyspermic fertilization highly improbable in sturgeons. These authors described fertilization process in sturgeons in detail and concluded that creation of cytoplasmatic projection in the sturgeon egg after fusion with the spermatozoon blocked polyspermic fertilization. This fact makes polyspermic fertilization as a formation mechanism of sturgeon autopolyploids with "abnormal" ploidy level at least questionable.

In contrast to polyspermic fertilization, suppression of the second mitotic division seems more plausible mechanism of formation of "abnormal" ploidy levels in sturgeon. Spontaneous diploidization of the maternal chromosome set followed by fertilization of this diploidized egg by haploid sperm is well known event in fish (Cherfas et al.,

1995; Borin et al., 2001; Centofante et al., 2001; Aegerter and Jalabert, 2004; Ezaz et al., 2004). Autopolyploids (mainly triploids) were also observed in different sturgeon species. Autopolyploids of *A. transmontanus* were reported by Drauch Schreier et al. (2011). Presence of triploid individuals was detected by Omoto et al. (2005) among progeny obtained from normally fertilized eggs of bester. In artificially produced progeny of *A. mikadoi* Zhou et al., 2011 described the occurrence of individuals with unusual ploidy for this species. By investigating of genome size Zhou et al. (2011) suggested these individuals to be triploids and some of them even tetraploids. Later on, Zhou et al. (2013) carried out the karyotype of previously observed triploids with 402 chromosomes. Hybrid origin of these individuals of *A. mikadoi* with "abnormal" ploidy levels seems improbable even though these authors did not provide any evidence for their autopolyploid origin.

In regard to the facts mentioned in previous paragraph and based on the results of our study (Havelka et al., 2013b; Chapter 3) where we confirmed autopolyploid origin of observed triploids of *A. ruthenus* and also based on confirmation of autopolyploid origin of triploid *A. baerii* male in Chapter 4, we assumed that spontaneous egg diploidization (usually by the junction of pronuclei in haploid egg as a result of suppressing the second meiotic division) might be possible way for formation of sturgeon autopolyploids. This general consideration was in contrast to Van Eenennaam et al. (1996) and Vasil'ev (2009) who stated that frequencies of spontaneous egg diploidization were close to zero in sturgeon.

Sturgeons still provide many unresolved questions and challenges for future research. First of all, their endangered status shows that protection and restoration interests must be conclusively placed above economical interests. Identification and evaluation of suitable nuclear markers for species and hybrids identification is necessary prerequisite for effective reintroduction of critically endangered acipenseriform species, nevertheless, to best of our knowledge, these markers have not been sufficiently identified. New techniques such "Next generation sequencing" probably provide new insight into the complicated sturgeon genome and allow identification not only of discriminative markers for species and hybrids identification but also sex related genes, quantitative trait loci and so on.

Also sturgeon aquaculture might play very important role in saving of wild sturgeon populations. Production of sturgeon juveniles for releasing programmes with respecting of all conservation principles and recommendations formulated at the 6th International Symposium on Sturgeons in Wuhan 2009 (Rosenthal et al., 2011) might help to preserve remaining wild sturgeon populations as was demonstrated in Northern America (Pikitch et al., 2005). Moreover, increasing production of caviar from aquaculture might ideally lead to the rapid decreasing of its prices on international market which could result in decreasing of profitability of poaching. It means that also in sturgeon aquaculture it is necessary to introduce new procedures like genetic screening of intraspecific and interspecific integrity of stocks, production of all female populations for more effective caviar production and many others.

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ENGLISH SUMMARY

Molecular aspects of interspecific hybridization of sturgeons related to polyploidy and *in situ* conservation

Miloš Havelka

Sturgeons (Chondrostei: Acipenseriformes) display markedly disjunct distributions with a wide occurrence in the northern hemisphere. Their unique benthic specialization, conserved morphology, evolutionary age, the variation in their basic diadromous life history, and the large public interest due to their near extinction or critically endangered status make sturgeons and paddlefishes one of the most interesting group of vertebrates. In addition to that, ploidy diversity of Acipenseriformes possessing three ploidy groups having ~ 120 chromosomes, ~ 240–270 chromosomes and ~ 360 chromosomes provides unique model for investigation of evolutionary processes which were going through the genome duplication events. Sturgeons are also notoriously known for their strong propensity to interspecific and intergeneric hybridization which can result in hybrids with various ploidy levels. All these facts make sturgeon genetics and cytogenetics a thriving but also complicated area for research.

In the present work, the role of genome duplication and functional reduction evens in evolution of sturgeon species as well as sturgeons' ploidy levels and ploidy relationships among Acipenseriformes were investigated using molecular markers. In addition to that, clarification of origin of abnormal ploidy levels and observation of segregation pattern of microsatellite alleles in the course of hybridization of polyploid sturgeon species were included into this study.

Using allele number evaluation at eleven microsatellite loci, evidence for functional diploidy, tetraploidy and hexaploidy in species possessing 120, 250 and 360 chromosomes, was provided. Moreover, observation of coexistence of diploid and tetraploid and/or tetraploid and octaploid allelic band patters in the same sturgeon genome confirmed that functional genome reduction played important role in recent sturgeon evolution and that this might be still an ongoing process. Based on observation of twelve alleles differing in size at microsatellite locus Atr 114, the remnant of the evolutionary dodecaploid genome conformation of *A. brevirostrum* was firstly observed by molecular data. However, with the respect to the determination of six functionally reduced microsatellite loci we confirmed that due to the recent functional genome reduction events, current functional ploidy status of *A. brevirostum* correspond to the hexaploidy.

Experimental crossbreeding of sturgeon species bearing different ploidy levels and chromosome numbers, followed by molecular investigation of obtained progeny, supported wildly accepted consideration about intermediate genome conformation of hybrids in respect to their parental species. Nevertheless, clarification of the origin of abnormal ploidy levels provided in this study showed that hybridization events did not have to be the only possible formation mechanism of abnormal ploidy levels of sturgeon species. Molecular investigation of six triploid specimens morphologically identified as *A. ruthenus,* in combination with five successive statistical analyses proved that one extra chromosome set added in genomes of these triploids originated from autotriploidization rather then from interspecific

hybridization. Similarly, confirmation of non hybrid origin of investigated triploid *A. baerii* male supported autopolyploidization as another mechanism of formation of sturgeons' abnormal ploidy levels.

Finally, detailed analyses of progeny obtained from experimental crossbreeding of spontaneous triploid of *A. baerii* male with *A. baerii* and *A. gueldenstaedtii* females provided clear evidence about fertility of this triploid in contrast to general belief in sterility or subfertility of triploid fishes due to interference of gametogenesis.

With regard to the all considerations and observations provided by this study we concluded that evolution of sturgeon species is still widely dynamic and ongoing process which might goes through the allopolyplodization as well as autopolyplidization events.

CZECH SUMMARY

Molekulární aspekty mezidruhové hybridizace jeseterovitých ryb
ve vztahu k polyploidii a *in situ* konzervaci

Miloš Havelka

Jednotlivé druhy jeseterů (nadřád: chrupavčití, řád: jeseteři) vykazují výraznou disjunkci areálu svého rozšíření na severní polokouli. Jejich potravní specializace, morfologie, evoluční historie a také status kriticky ohrožených druhů, v některých případech dokonce druhů na pokraji vyhynutí, dělá z jeseterů a veslonosů jednu z nejzajímavějších skupin obratlovců na naší planetě. Mimoto, ploidní diverzita jeseterovitých ryb, reprezentovaná druhy s ~ 120 chromozómy, 240–270 chromozómy a jedním druhem s ~ 360 chromozómy v jádrech svých buněk, poskytuje unikátní model pro studium evolučních procesů jdoucích skrze celogenomové duplikační události. Jeseterovité ryby jsou také notoricky známé pro svoji výraznou náchylnost k mezidruhové hybridizaci, která může mít za následek tvorbu hybridů s různými úrovněmi ploidie. Všechny tyto skutečnosti dělají ze studia genetiky a cytogenetiky jeseterovitých ryb prosperující, ale také obtížnou oblast vědeckého poznání.

V této práci byl za pomoci zvolených molekulárních márkrů studován význam celogenomových duplikačních a funkčně redukčních událostí v evoluci jeseterů, jejich ploidní úrovně a ploidní vztahy uvnitř tohoto řádu chrupavčitých ryb. Dále byl studován původ abnormálních ploidních úrovní jeseterovitých ryb a také vzor segregace mikrosatelitních alel při mezidruhové hybridizaci polyploidních druhů jeseterů.

Pomocí vyhodnocování počtu alel na jedenácti mikrosatelitních lokusech bylo prokázáno funkčně diploidní, respektive tetraploidní a hexaploidní uspořádání genomu jeseterovitých ryb se ~ 120 chromozómy, 240–270 chromozómy a ~ 360 chromozómy. Navíc, koexistence diploidního a tetraploidního nebo tetraploidního a oktaploidního alelového vzoru vždy u jednoho ze studovaných druhů potvrdila, že funkční genomové redukce hrály významnou roli v nedávné evoluční historii jeseterů a že tento proces nemusí být zcela ukončen. Na základě nalezení až dvanácti různých alel na lokusu Atr 114 byl poprvé pozorován pozůstatek evoluční dodekaploidie jesetera krátkorypého na molekulární úrovni. Nicméně, s ohledem k souběžnému nalezení šesti funkčně redukovaných mikrosatelitních lokusů bylo v této studii potvrzeno současné funkčně hexaploidní uspořádání genomu tohoto druhu.

Experimentální křížení druhů jeseterů s různými úrovněmi ploidie a s rozdílným počtem chromozómů, následované molekulární analýzou získaného potomstva, potvrdilo široce akceptovaný předpoklad o intermediálním uspořádání genomu hybridních jedinců s ohledem na původní rodičovské druhy. Nicméně, ověření původu abnormálních ploidií také realizované v této studii ukázalo, že hybridizace nemusí být nutně jediným mechanizmem formování abnormálních plodních úrovní u jeseterovitých ryb. Molekulární analýza šesti funkčně triploidních jedinců, morfologicky určených jako jeseter malý, v kombinaci s pěti statistickými metodami dokázala, že jedna přidaná sádka chromozomů v genomu těchto triploidů pochází spíše z autopolyploidizace než z mezidruhové hybridizace. Obdobně, potvrzení nehybridního původu triploidního samce jesetera sibiřského podpořilo předpoklad o autopolyploidizaci jako dalším možném mechanizmu formování abnormálních ploidních úrovní jeseterovitých ryb.

Konečně, podrobná analýza potomstva získaného z experimentálního křížení spontánně triploidního samce jesetera sibiřského se samicemi jesetera sibiřského a ruského poskytla jasný důkaz o plodnosti tohoto triploidního jedince, a to i navzdory obecnému předpokladu sterility triploidních ryb v důsledku narušení gametogeneze.

S ohledem na všechny předpoklady a pozorování popsané v této studii jsme dopěli k závěru, že evoluce jeseterovitých ryb je stále dynamicky probíhající proces, který může probíhat skrze allopolyploidizační stejně tak jako autopolyploidizační události.

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Havelka, M., Hulák, M., Bytyutskyy, D., Rábová, M., Ráb, P., Rodina, M., Gela, D., Flajšhans, M., 2012. Application of molecular and cytogenetic markers in sturgeon aquaculture: a case study on sterlet (*Acipenser ruthenus*) and Russian sturgeon (*Acipenser gueldenstaedtii*) with odd ploidy levels. The 11th International Symposium on Genetics in Aquaculture, Abstract Book. Auburn University Hotel and Dixon Conference Center, Auburn, Alabama, USA, p. 65.

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TRAINING AND SUPERVISION PLAN DURING STUDY

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The XI International Symposium on Genetics in Aquaculture, 25–29 June 2012, Auburn University Hotel and Dixon Conference Center, Auburn, Alabama, USA. (oral presentation)	2012
World Aquaculture Society Conference AQUA 2012, 1–5 September 2012, Prague Congress Centre, Prague, Czech Republic. (poster presentation)	2012

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Bc. students supervision		Year
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Hana Šachlová	Impact of interspecific hybridization on sturgeons´ fitness	2013

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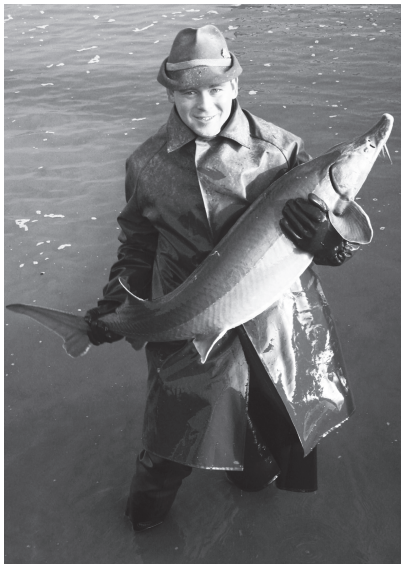
Jaroslava Lidová	Molecular aspects of interspecific hybridization of sturgeon species	2011
Dagmar Hucková	Application of molecular markers for studying of fish biodiversity: A case study on wild grayling populations	2012
Anastasija Dujkova	Application of molecular markers for studying of fish biodiversity: A case study on aquaculture grayling populations	2012

CURRICULUM VITAE



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