

Chapter 2

Cytogenetics as a Tool for an Exploration of *A. sturio* Status Within Sturgeons

Francesco Fontana

Abstract A karyotype analysis carried out on the European sturgeon, *Acipenser sturio*, revealed a chromosome number of $2n = 122 \pm 3$. A representative karyotype of 122 chromosomes was composed of 66 meta- and submetacentrics, 56 acrocentrics and microchromosomes. The telomeric sequence repeat (TTAGGG) $_n$ detected by fluorescent in situ hybridization (FISH) was localized at the telomeres of all chromosomes. The ribosomal DNA (rDNA) genes were detected by FISH with a digoxigenin-labelled probe for 28S rDNA in the telomeric regions of six chromosomes. The 5S rDNA was found in the interstitial region of a small meta-centric pair. The close association between the two rDNA families detected by simultaneous two-colour in situ hybridization (sim-FISH) supports the hypothesis that the *A. sturio* karyotype is primitive. The results are discussed in relation to morphophysiological affinities between *A. sturio* and *A. oxyrinchus*.

Glossary

Cytochrome b (cyt b)

A protein component of respiratory chain, involved in electron transport and the production of ATP.

Fluorescent in situ hybridization (FISH)

Cytogenetic technique used to detect and localize the presence of specific DNA sequences on chromosomes. FISH uses fluorescent probes that only bind to the chromosome parts with high sequence similarity.

F. Fontana (✉)

Department of Biology and Evolution, University of Ferrara, Via L. Borsari 46,
44100 Ferrara, Italy
e-mail: francesco.fontana@unife.it

Haplotype	An haplotype is a combination of DNA sequences on one chromosome which tend to be inherited together.
<i>Hind</i> III and <i>Pst</i> I satellite DNA	Two satellite DNA families (respectively identified by restriction enzymes of <i>Haemophilus indicus</i> and <i>Providencia stuartii</i>) frequently studied in sturgeons.
Satellite DNA	Short and highly repeated DNA sequences. Most satellite DNA is localized to the telomeric or the centromeric region of the chromosomes. The nucleotide sequence of the repeats is fairly well conserved among species.
sim-FISH	Simultaneous detection of the two gene families by different staining of two different gene probes. The simultaneous visualization of the two colours is obtained with an epifluorescence microscope equipped with special band filters.
Telomeric sequence	A region of repetitive DNA sequences located at the end of a chromosome, which protects the end of the chromosome from being cut at the end of linear DNA replication.
28S, 16S, 5S rDNA	Regions of DNA respectively codifying for 28S, 16S and 5S ribosomal RNAs. They are composed of tandem repeats of sequences.

2.1 Introduction

Within Acipenseridae taxonomy, a highly debated issue is the relationship between the two species *Acipenser sturio* (European sturgeon) and *A. oxyrinchus* (Atlantic sturgeon) (see Magnin 1962). Based on detailed morphological and anatomical studies, Magnin (1962) and Magnin and Beaulieu (1963) stated that the two species, although very close, should be considered as separate ones. Other authors (Artyukhin and Vecsei 1999) concluded on the same bases that the differences were too small to support the status of separate species, and that therefore the two taxa had to be considered as geographically separated populations of the same species.

Recently, several molecular biology data show that *A. sturio* and *A. oxyrinchus* not only are closely related species, but belong to a group significantly separated

from the other Acipenseridae, which represent the most ancestral sturgeon group. The phylogenetic data obtained by molecular analyses of partial sequences of three mitochondrial gene regions (respectively the cytochrome b region and fragments of 12S and 16S rDNA from 22 species of Acipenseridae) (Birstein and DeSalle 1998) show that *A. sturio* and *A. oxyrinchus* represent a separate evolutionary lineage within Acipenseridae. These results are supported by analyses of other sequences of mitochondrial genes in a higher number of species (Birstein et al. 2002). Moreover, molecular phylogeny analyses based on cytochrome b sequences support the separation of these two species from the other 13 species belonging to the subfamily Acipenserinae (Ludwig et al. 2001). Also, recent studies on satellite DNA distribution in the same 15 species of Acipenserinae by Southern blot hybridization show that *Pst*I satellite DNA sequences are present in all species analyzed, while *Hind*III satellite DNA sequences are present in all species except *A. sturio* and *A. oxyrinchus* (Robles et al. 2004). A more detailed analysis of *Pst*I satellite DNA in the same 15 species showed a cladistic association of *A. sturio* and *A. oxyrinchus* sequences, and very similar results were also obtained for 5S rDNA (Robles et al. 2005). Apparently, the two species represent a single clade separated from other Acipenserinae species (Robles et al. 2005). A very recent revision of molecular phylogeny of 20 Acipenseriformes species (Krieger et al. 2008), based on combined mitochondrial DNA sequences, shows that *A. sturio* and *A. oxyrinchus* are phylogenetically ancestral species, early branching from the common ancestor of other sturgeons. These data support previous palaeontological findings (Nesov and Kaznyshkin 1983), which consider *A. sturio* and *A. oxyrinchus* as the most primitive species within the genus *Acipenser*.

Within this complex issue, a detailed knowledge of *A. sturio* cytogenetics could be relevant to clarify the evolution and phylogeny of this species in comparison to the other sturgeons.

2.2 Materials and Methods

Tissue fragments of three living individuals of *A. sturio* were sampled in 1998 for cytogenetic analyses from the Gironde estuarine population, and sent to the Cytogenetics Laboratory at the Department of Biology and Evolution, University of Ferrara (Italy) in a refrigerated container. From these fragments, primary cell lines were established from fibroblast fin culture using the technique described by Fontana et al. (1997). The cell lines were maintained in liquid nitrogen at -70°C , to ensure euploidy along time, as verified by regular use in cytogenetic investigations (Lanfredi et al. 2001; Fontana et al. 2003). When required, fibroblasts were recovered from liquid nitrogen, transferred to sterile plastic flasks and allowed to grow to confluence. Cells were then collected, hypotonically treated and fixed for slide preparations of metaphases (Fontana et al. 1997).

Fluorescent in situ hybridization (FISH) was employed on *A. sturio* fibroblasts to localize the telomeric sequence by the deoxynucleotide (TTAGGG) $_n$ oligomer,

according to standard techniques (Appligene Oncor, Illkirch, France). The two ribosomal DNA probes for FISH were derived from the genomic DNA of *A. naccarii*. The 28S rDNA probe was composed of two different fragments (approximately 400- and 700-bp long) of the coding region, obtained by two sets of previously described primers (Zardoya and Meyer 1996). The 5S probe, derived from genomic DNA of *A. naccarii*, was composed of a 230-bp fragment, cloned in pMOS-blue vector (Amersham Int., USA) and digoxigenin-labelled using the PCR DIG probe Synthesis Kit (Boehringer, Mannheim, Germany). To simultaneously detect the distribution of the two gene families, a simultaneous two-colour FISH (sim-FISH) was performed. The ribosomal sequences 28S probe was labelled with biotin-16-dUTP (Boehringer), the 5S with digoxigenin-11-dUTP (Boehringer), according to the manufacturer's instructions. Slides were examined using a Leitz epifluorescence microscope with triple band filter for simultaneous visualization of the three colours.

The meaning of the most common genetic expressions is given as a glossary at the end of the chapter.

2.3 Results

The mitotic karyotypes of fibroblast cell lines derived from three *A. sturio* individuals of unknown sex were examined. At least 30 metaphase plates were scored for each cell line. Analyses of all data indicate that the mean chromosome number of *A. sturio* is $2n = 122 \pm 3$. Among all available metaphase plates, one was chosen for karyotype reconstruction because it was isolated, round and surrounded by a faint cytoplasmic residue which protected it from loss or gain of chromosomes. This representative plate, with 122 chromosomes, is shown in Fig. 2.1. Because of their gradual size and morphology, the chromosomes are approximately gathered into two groups, arranged in order of decreasing length: the meta- submetacentrics (33 pairs), acrocentrics and microchromosomes (56 in total). The chromosomes belonging to the second group were individually aligned in the karyotype because they can not be paired. The fundamental number (that is the number of chromosome arms) is therefore 188.

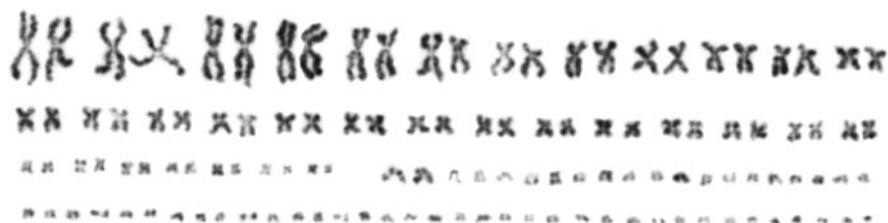


Fig. 2.1 Karyotype of *Acipenser sturio* ($2n = 122$). The meta- and submetacentric chromosomes are aligned in order of decreasing size, followed by acrocentrics and microchromosomes

The telomere signals, detected by FISH as definite spots, are located at both ends of each chromosome arm. No hybridization signal is observed at the interstitial sites (Fig. 2.2a). The *in situ* hybridization with the 28S rDNA probes reveal clear signals of different intensities, interspersed within the telomeric regions of six chromosomes (Fig. 2.2b). After hybridization with the 5S probe, two intense fluorescent signals are observed in the middle region of a pair of small chromosomes (Fig. 2.2c). The results of sim-FISH with biotin-labelled 28S (green hue) and digoxigenin-labelled (red hue) 5S rDNA probes on chromosomes counterstained with DAPI (blue hue) are shown in Fig. 2.2d. The signals of 28S probe, which show different intensities, are localized within the telomeric regions of six chromosomes. There

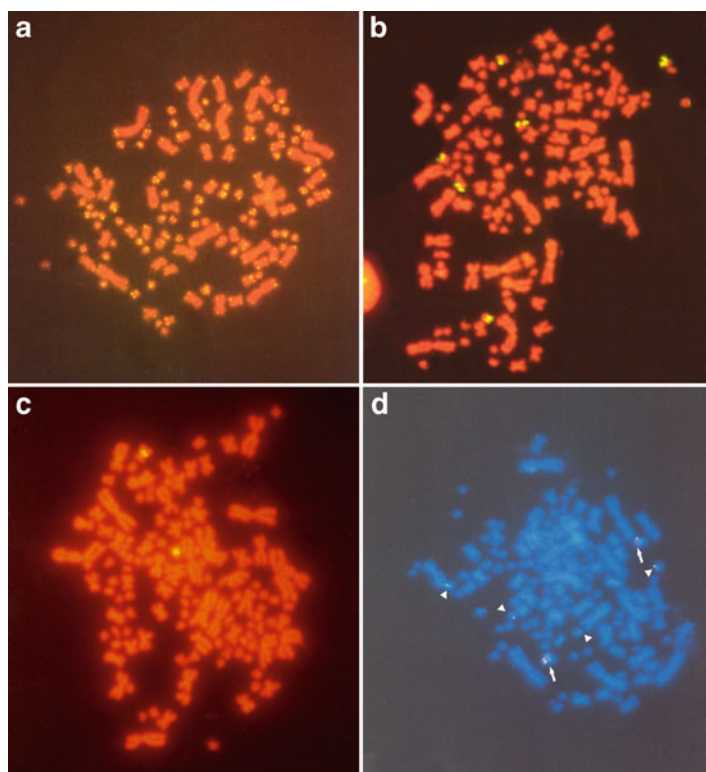


Fig. 2.2 Metaphases of *A. sturio*. **a** FISH with biotin-labelled (TTAGGG)_n oligomers, counterstained with propidium iodide. **b** FISH of the 28S rDNA probe detected with digoxigenin and counterstained with propidium iodide. **c** FISH of the 5S rDNA probe detected with digoxigenin and counterstained with propidium iodide. **d** simultaneous two-colour fluorescent *in situ* hybridization. The micrographs were taken with a triple band filter allowing the simultaneous visualization of the DAPI-stained chromosomes (*blue colour*), the hybridization sites of the 18S–28S (*green*, fluorescein) and the 5S (*red*, Texas Red) rDNA probes. In the loci where the major and minor rDNA signals overlap, the resulting colour is *yellow* (*arrows*). *Arrowheads* show the major rDNA signals

are two 5S-positive regions on one chromosome pair, apparently overlapping two 28S-positive regions.

2.4 Discussion

The karyotype of the Acipenseridae family is characterized by a very high number of chromosomes, about half of which are microchromosomes. The sturgeons may be karyotypically divided into three groups: the first with ~120 chromosomes (from 112 to 146), the second with ~250 chromosomes (from 240 to 270) and the third containing only one species with 372 chromosomes (Fontana et al. 2008a) (for reviews see the Web site <http://www.unife.it/dipartimento/biologia-evoluzione/progetti/geneweb>). The karyotype of *A. sturio*, the European sturgeon ($2n = 116 \pm 4$), was first described by Fontana and Colombo (1974), and later as ($2n = 121 \pm 3$) by Tagliavini et al. (1999). These data are supported by the present results ($2n = 122 \pm 3$), which confirm that the species belongs to the 120-chromosome group. The telomeric sequence (TTAGGG) $_n$ repeats detected by FISH are scattered in the end regions of chromosome arms. The absence of interstitial telomeric signals suggests that no recent chromosome rearrangements have occurred in this species. A similar pattern of telomeric signal distribution was observed in all sturgeon species except *A. gueldenstaedtii*, in which two chromosomes are entirely marked with blocks of repeating telomeric sequences (Fontana et al. 1998). Assuming that the ancestral number of 5S rDNA chromosome pairs is 1, the 120-chromosome group could represent the primitive (plesiomorphic) condition among sturgeons, while the 250-chromosome group and the 372 one (respectively with two and three pairs of 5S rDNA) could represent the derived (or apomorphic) condition. The primitive position of *A. sturio* within the genus *Acipenser* is also supported by the fact that the species belongs to the 120-chromosome group.

The karyotype association (synteny) between major and minor rDNA genes repeatedly appeared and disappeared during the evolution of eukaryotic genomes. Its widespread presence in lower eukaryotes has been interpreted as an ancestral condition (Drouin and Moniz de Sá 1995; Martins and Galetti 1999), but other authors maintain that in genome evolution the ancestral condition is the separation of the two rDNA gene families on different chromosome pairs (Pendás et al. 1994; Martínez et al. 1996). Given the ancestral position of Acipenseriformes within fishes, our results support the hypothesis that synteny of rDNA genes represents the ancestral condition within fishes and most probably also in vertebrates.

The main karyotype features, such as chromosome number, distribution of telomeric regions, major (28S) and minor (5S) rDNA, are shared not only by *A. sturio* and *A. oxyrinchus*, but also by other species belonging to the 120-chromosome group, such as *A. ruthenus*, *A. stellatus* and *Huso huso* (Fontana 2002). Thus, the above cytogenetic features are scarcely informative tools to discriminate *A. sturio* and *A. oxyrinchus* from other sturgeon species, but the results obtained by FISH with satellite DNA probes are useful for this purpose. *A. sturio*

and *A. oxyrinchus* do not show any signal with *Hind*III satellite DNA probe, contrary to *A. stellatus*, *A. ruthenus* and *Huso huso*, and to 240-chromosome species such as *A. naccarii*, *A. gueldenstaedtii*, *A. baeri* and *A. transmontanus* (Lanfredi et al. 2001; Fontana et al. 2008b). These cytogenetic results are in agreement with previous molecular data obtained by Southern-blot hybridization (Robles et al. 2004). *A. sturio* and *A. oxyrinchus* can also be discriminated from all other sturgeon species by *Pst*I satellite DNA probes: they do not show any hybridization signal, while *A. stellatus* and *A. gueldenstaedtii* show hybridization (Fontana et al. 2008b). The most relevant result is that *A. sturio* and *A. oxyrinchus* react in the same way to both satellite DNA probes. These data support the close association of the two species in a clade that separated early from the other sturgeons (Robles et al. 2004).

Overall, the karyological, cytogenetic and molecular data support the hypothesis that *A. sturio* and *A. oxyrinchus* are very similar and represent a sister clade in comparison to all other sturgeon species. Moreover, the molecular data favour the hypothesis that *A. sturio* and *A. oxyrinchus* are the oldest clade within the genus *Acipenser* (Ludwig et al. 2000). It is reasonable to presume that about 90 M years ago there was an ancestral Atlantic *Acipenser* form (Birstein and Doukakis 2000), which later divided into two forms (presumably *A. sturio* and *A. oxyrinchus*), now surviving on the opposite sides of the North Atlantic: therefore, their interbreeding is presently prevented. Detailed data on geographical distribution of mitochondrial DNA haplotypes show the presence of *A. oxyrinchus* in Northern Europe (Baltic Sea), presumably during the Middle Ages (Ludwig et al. 2002). More recent archaeozoological and paleontological studies witness that *A. oxyrinchus* inhabited the French Atlantic region at the end of the Neolithic Age, about 5,000 years ago (Desse-Berset 2009). With regard to the time elapsed since the separation of the two species, two hypotheses can be advanced. If the separation occurred recently, i.e., during climatic changes in Pliocene and Pleistocene (Choudhury and Dick 1998), then there was not enough time for the two species to significantly differentiate. On the contrary, if the separation occurred a long time ago, their high degree of similarity could be due to the slow genome evolution of *Acipenseriformes* (Gardiner 1984; Krieger and Fuerst 2002), as also supported by cytogenetic studies on species with the same ploidy degree which show a highly conserved genome (Fontana et al. 2001).

References

- Artyukhin E, Vecsei P (1999) On the status of Atlantic sturgeon: conspecificity of European *Acipenser sturio* and the North American *Acipenser oxyrinchus*. *J Appl Ichthyol* 15:35–37
- Birstein VJ, DeSalle R (1998) Molecular phylogeny of *Acipenserinae*. *Mol Phylogenet Evol* 9:141–155
- Birstein VJ, Doukakis P (2000) Molecular analysis of *Acipenser sturio*, L., 1758 and *A. oxyrinchus* Mitchill, 1815: a review. *Bol Inst Esp Oceanogr* 16:61–73
- Birstein VJ, Doukakis P, DeSalle R (2002) Molecular phylogeny of *Acipenseridae*: nonmonophyly of *Scaphirhynchidae*. *Copeia* 2:287–301

- Choudhury A, Dick TA (1998) The historical biogeography of sturgeons (Osteichthyes: Acipenseridae): a synthesis of phylogenetics, palaeontology and palaeogeography. *J Biogeogr* 25: 623–640
- Desse-Berset N (2009) First archaeozoological identification of Atlantic sturgeon (*Acipenser oxyrinchus* Mitchell 1815) in France. *CR Palevol* 8:717–724
- Drouin G, Moniz de Sá M (1995) The concerted evolution of 5S ribosomal genes linked to the repeat units of other multigene families. *Mol Biol Evol* 12:481–493
- Fontana F (2002) A cytogenetic approach to the study of taxonomy and evolution in sturgeons. *J Appl Ichthyol* 18:226–233
- Fontana F, Colombo G (1974) The chromosomes of Italian sturgeons. *Experientia* 30:739–742
- Fontana F, Rossi R, Lanfredi M, Arlati G, Bronzi P (1997) Cytogenetic characterization of cell lines from three sturgeon species. *Caryologia* 50:91–95
- Fontana F, Lanfredi M, Chicca M, Aiello V, Rossi R (1998) Localization of the repetitive telomeric sequence (TTAGGG)_n in four sturgeon species. *Chromosome Res* 6:303–306
- Fontana F, Tagliavini J, Congiu L (2001) Sturgeon genetics and cytogenetics: recent advancements and perspectives. *Genetica* 111:359–373
- Fontana F, Lanfredi M, Congiu L, Leis M, Chicca M, Rossi R (2003) Chromosomal mapping of 18S–28S and 5S rRNA genes by two-colour fluorescent in situ hybridization in six sturgeon species. *Genome* 46:473–477
- Fontana F, Congiu L, Mudrak VA, Quattro JM, Smith TIJ, Ware K, Doroshov SI (2008a) Evidence of hexaploid karyotype in shortnose sturgeon. *Genome* 51(2):113–119. doi:10.1139/G07-112
- Fontana F, Lanfredi M, Kirschbaum F, Garrido-Ramos MA, Robles F, Forlani A, Congiu L (2008b) Comparison of karyotypes of *Acipenser oxyrinchus* and *A. sturio* by chromosome banding and fluorescent in situ hybridisation. *Genetica* 132:281–286. doi:10.1007/s10709-007-9171-4
- Gardiner BG (1984) Sturgeons as living fossils. In: Eldredge N, Stanley SM (eds) *Living fossils*. Springer, New York, pp 148–152
- Krieger J, Fuerst PA (2002) Evidence for a slowed rate of molecular evolution in the Order Acipenseriformes. *Mol Biol Evol* 19:891–897
- Krieger J, Hett AK, Fuerst PA, Artyukhin E, Ludwig A (2008) The molecular phylogeny of the order Acipenseriformes revisited. *J Appl Ichthyol* 24(suppl 1):36–45
- Lanfredi M, Congiu L, Garrido-Ramos MA, De La Herrán R, Leis M, Chicca M, Rossi R, Tagliavini J, Ruiz Rejón C, Ruiz Rejón M, Fontana F (2001) Chromosomal location and evolution of a satellite DNA family in seven sturgeon species. *Chromosome Res* 9:47–52
- Ludwig A, May B, Debus L, Jenneken I (2000) Heteroplasmy in the mtDNA control region of sturgeon (*Acipenser*, *Huso* and *Scaphirhynchus*). *Genetics* 156:1933–1947
- Ludwig A, Belfiore NM, Pitra C, Svirsky V, Jenneken I (2001) Genome duplication events and functional reduction of ploidy levels in sturgeon (*Acipenser*, *Huso* and *Scaphirhynchus*). *Genetics* 158:1203–1215
- Ludwig A, Debus I, Lieckfeldt D, Wirgin I, Benecke N, Jenneken I, Williot P, Waldman JR, Pitra C (2002) When the American sea sturgeon swam east. *Nature* 419:447–448
- Magnin E (1962) Recherches sur la systématique et la biologie des Acipenseridés, *Acipenser sturio* L., *Acipenser oxyrinchus* Mitchell et *Acipenser fulvescens* Raf. *Ann Stat Cent Hydrobiol Appl* 9:7–242
- Magnin E, Beaulieu G (1963) Étude morphométrique comparée de l'*Acipenser oxyrinchus* Mitchell du Saint-Laurent et l'*Acipenser sturio* Linné de la Gironde. *Le Naturaliste Canadien* 40:5–38
- Martínez JL, Morán P, García-Vázquez E, Pendás AM (1996) Chromosomal localization of the major and 5S rRNA genes in the European eel (*Anguilla anguilla*). *Cytogenet Cell Genet* 73: 149–152
- Martins C, Galetti PM Jr (1999) Chromosomal localization of 5S rDNA genes in *Leporinus* fish (Anostomidae, Characiformes). *Chromosome Res* 7:363–367
- Nesov LA, Kaznyshkin MN (1983) New sturgeons from the Cretaceous and Paleogene of the USSR. In: Menner VV (ed) *Contemporary problems of paleoichthyology*. Nauka, Moscow, pp 68–76

- Pendás AM, Morán P, Freije JP, Garcia-Vazquez E (1994) Chromosomal mapping and nucleotide sequence of two tandem repeats of Atlantic salmon. *Cytogenet Cell Genet* 67:31–36
- Robles F, De La Herrán R, Ludwig A, Ruiz Rejón C, Ruiz Rejón M, Garrido-Ramos MA (2004) Evolution of ancient satellite DNAs in sturgeon genomes. *Gene* 338:133–142
- Robles F, De La Herrán R, Ludwig A, Ruiz Rejón C, Ruiz Rejón M, Garrido-Ramos MA (2005) Genomic organization and evolution of the 5S ribosomal DNA in the ancient fish sturgeon. *Genome* 48:18–28
- Tagliavini J, Williot P, Congiu L, Chicca M, Lanfredi M, Rossi R, Fontana F (1999) Molecular cytogenetic analysis of the karyotype of the European Atlantic sturgeon, *Acipenser sturio*. *Heredity* 83:520–525
- Zardoya R, Meyer A (1996) Evolutionary relationships of the Coelacanth, Lungfishes, and Tetrapods based on the 28S ribosomal RNA gene. *Proc Natl Acad Sci USA* 93:5449–5454

Biology and Conservation of the European Sturgeon

Acipenser sturio L. 1758

The Reunion of the European and Atlantic Sturgeons

Williot, P.; Rochard, E.; Desse-Berset, N.; Kirschbaum, F.; Gessner, J. (Eds.)

2011, XXVIII, 668 p., Hardcover

ISBN: 978-3-642-20610-8