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Low Danube Sturgeon Identification Using DNA Markers

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1. Introduction

Sturgeons represent a class of fish with a distinctive importance, both from a scientific and a commercial point of view. This group of fish appeared 200 million years ago (Bemis *et al.*, 1997) and the early diversification of the species took place in a geographical area currently situated in Asia. Since these are some of the oldest fish species in the world, having survived several mass extinction events, authentic "living fossils" with a slow evolution, sturgeons may constitute a study model for the development of all vertebrates. The commercial importance of these species is due to the value of the products obtained from them, especially caviar, which is considered a gourmet delicacy. Unfortunately, due to their economic importance, these species have been overly exploited through overfishing and poaching. As a result, nowadays they are on the brink of extinction.

General knowledge about sturgeons has been available starting with the last decades of the nineteenth century. In the twentieth century, the interest in sturgeons spread and currently there is a continuous request for new information about the physiology, genetics, phylogeny and evolution history of the species within the *Acipenseriformes* order. The reasoning for this continued interest is the fact that these species are truly endangered, have an increasingly limited distribution area and a continually decreasing number of individuals.

The *Acipenseriformes* order includes two families: *Acipenseridae* (sturgeons proper) and *Polyodontidae* (paddlefish). The distribution of the current 26 species (27, in accordance with Bemis & Kynard, 1997) belonging to the *Acipenseriformes* order is limited to the northern hemisphere (Rochard *et al*, 1991). It has been estimated that approximately half of the sturgeon species are found in Europe (especially in the Pontic-Caspian region), a third of North America and the remainder in eastern Asia and Siberia (Billard & Lecointre, 2001). Sturgeons are distributed on a wide-spread geographical territory, ranging from subtropical to subarctic waters, from North America to Eurasia, in rivers, lakes, coastal waters and inland seas. These species can be found on the European coast of the Atlantic, in the Mediterranean Sea basin, in the Black Sea, the Caspian Sea, the Azov Sea and the rivers in north-western Russia flowing into the Arctic Ocean (Ob, Yenisei, Lena, Kolyma), in Central Asian rivers (Amu Darya and Syr Darya) and in the Baikal Lake. On the Pacific Ocean coast these fish may be encountered in the Amur River, along the Russian-Chinese border, in the hydrographic basin of the Yangtze River and of other rivers in north-eastern China.

Currently, the greatest diversity of *Acipenseridae* species can be found in the Pontic-Caspian region. Some of the sturgeon species from this area possess certain obvious morphologic traits which set them apart from all other sturgeon species, such as the elongated rostrum of the *Acipenser stellatus* species. The Pontic-Caspian region has been extremely unstable during the last 150 million years, a period during which the scientists hypothesise that the *Acipenseridae* diversification had occurred. These changes refer to major variations of the seawater level, the transformation of great water expanses such as the Black Sea from fresh water lakes into enclosed salt-water environments, as well as important modifications regarding river flows along with the formation of mountains. The Black Sea was repeatedly connected to and disconnected from the Caspian and Aral Seas. Thus, the link between the great diversity of *Acipenseridae* in this region and its extremely complex evolutionary history is more than evident.

Most of the sturgeon species are on the brink of extinction. The majority of specialists believe that the changes that have intervened in the habitat of these fish represent the main causes of decline in the sturgeon populations. Among the dramatic anthropogenic factors influencing these fish the most important are overfishing and poaching, pollution and destruction of breeding sites, construction of dams which limit the migration towards spawning sites.

As a consequence of this situation, protection measures of these extremely valuable species have been adopted on an international level. Thus, all these species have been included on the red list of threatened species initiated by IUCN (International Union for Conservation of Nature), and trading with products originating from these fish is controlled by CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora).

2. General characterization of sturgeons

The classification of sturgeons in accordance with the traditional systematic is the following: regnum *Animalia*, superphylum *Vertebrata*, subphylum *Gnatosthomata*, superclass *Pisces*, class *Osteichtyes* (bony fish), subclass *Actinopteryngi,* superorder *Chondrostei*, order *Acipenseriformes*. The majority of authors agree with the traditional conception that the order *Acipensiformes* consists of two families, *Acipenseridae* and *Polyodontidae*. The *Acipenseridae* family includes two subfamilies *Acipenserinae* and *Scaphirhynchinae* and four genera (*Acipenser*, *Huso*, *Scaphirhynchus* and *Pseudoscaphirhynchus)*, while the *Polyodontidae* family has two genera (*Polyodon* and *Psephurus*), each with only one species (Rochard *et al.*, 1991; Bemis *et. al.*, 1997).

2.1 Morphological characteristics

The species of the *Acipenseriformes* order are characterized by their large or very large size. Sturgeons have an elongated body with a thicker foreside, large head, small eyes and fins oriented towards the posterior. In this group of fish the scales and lateral line are missing. The dorsal part of the body has a varied colouring, ranging from grey, brown, dark blue to close to black; the colouring becomes less intense on the ventral side. The majority of the species has a light colouring or even white on their ventral side.

Sturgeons possess certain relict characteristics, which set them apart from other fish and prove their extremely old origin. They have a cartilaginous endoskeleton, heterocercal

caudal fin, low pectoral fins, and undifferentiated vertebrae, with ganoid scales only in the caudal part of their body. The rest of the body is either naked or covered in five longitudinal rows of bony plates (scutes). The scutes are located one dorsally, two laterally and two ventrally, among these rows small scutes are set irregularly. The skeleton is mostly cartilaginous, with partial ossification only in the case of the cranial arch and maxillary. The cranium is elongated and forms a conical, spatulated rostrum with four ventral sensory barbels used for capturing food, and the inferior mouth is toothless and has thick lips. The endocranium is massive, without an interorbital septum and with few endochondral ossifications. The palatoquadrate cartilages are united on the median line and they are not directly attached to the cranium. The premaxillary is sealed to the maxillaries, which in their turn are sealed to the palatoquadrates and they are not independently mobile. The dermal bones of the cranium are hard. The vertebral bodies are not differentiated; the first vertebrae are attached to the cranium. The mouth, which is situated ventrally, is protrusible. The teeth are reduced or lacking, the branchiostegal rays are also missing. The intestine has a spiral valve and it communicates with the air bladder. The gills are protected by opercula or gill covers, the pectoral fins extend laterally from the body in a generally horizontal orientation and the caudal fin has two asymmetrical lobes. The number of basal elements in the fins is smaller than that of the rays. The operculum consists of a subopercle and a rudimentary preopercle. Some of the characteristics of the *Acipenseriformes* are primitive, while others indicate degeneracy, while others yet are specialized.

2.2 Sturgeon reproduction

Sturgeons migrate for reproduction and feeding. In what regards their life cycle, two migration models have been described (Bemis & Kynard, 1997):

1. **diadromous:** migrating from sea water to fresh water, and in reverse.
   The majority of the *Acipenseriformes* are diadromous species and exhibit two types of migration behaviour:
   a. **anadromous –** spend most of their life cycle in their feeding areas in the sea and migrate to fresh water areas for breeding. The two species of the *Huso* genus and the majority of the species of the *Acipenser* genus are anadromous.
   b. **amphidromous** – in fresh water – the breeding phase of their life cycle occurs in fresh water, while the feeding and growth phases occur during the migration to sea;
2. **potamodromous**: the fish migrate within the river/lake (McDowall 1988, 1992).
   The moment of reproduction for the *Acipenseridae* varies widely. Sturgeons breed during any of the seasons depending on water temperature and water flow velocity. In addition, the distance covered by sturgeons in their migration from the feeding grounds in the sea to the breeding areas in the rivers varies and is correlated with the fact that the existence of adequate breeding areas is essential for the success of breeding. The sites preferred by females for spawning are areas with a hard substrate consisting in gravel and rocks, with numerous crevices and with moderate currents. The annual success of breeding is difficult to estimate; in fact there may be no success if the water flow velocity is too high, whether it is due to natural phenomena, or the controlled release of water from dams and reservoirs. Water currents which are too strong may totally compromise or significantly reduce the success of breeding.

An interesting trait of sturgeon reproduction is the fact that these fish repeatedly use the same breeding site. The constancy and "fidelity" towards the breeding areas have been emphasised in the sturgeon populations in the Danube. This constancy and "fidelity" have led to the hypothesis that there are several sturgeon subpopulations in this river. For now there is no direct proof to this effect. However, if it is demonstrated that the sturgeons possess this strong instinct to maintain their breeding territories, this may explain the existence of the various types (morphs) within the *Acipenseridae* species.

2.3 Sturgeons species in the Lower Danube

Measuring 2857 km in length, the Danube is the second longest river in Europe. The river is divided into three regions: the Upper Danube (from the river's source in the Black Forest in Germany to Vienna) – 890 km, Middle Danube (from Vienna to the Iron Gates I Dam) – 993 km and Lower Danube (from Iron Gates I to its flow into the Black Sea) – 942 km.

Currently three diadromous species of sturgeons may be found in the Lower Danube basin and in the Black Sea - *H. huso* (beluga sturgeon), *A. stellatus* (stellate sturgeon) and *A. gueldenstaedtii* (Russian sturgeon) and one potamodromous species – *A. ruthenus* (sterlet) (Bacalbaşa-Dobrovici, 1999). The other two species that used to be found in this area, *A. sturio* (common European sturgeon) and *A. nudiventris* (ship sturgeon), are considered extinct, although, by way of exception, ship sturgeons are still being caught in the Danube basin (Kynard *et al.*, 2002).

Huso huso (Linaeus, 1758) (Beluga Sturgeon)

Distribution: in the Black Sea, Azov Sea, Caspian Sea and the rivers tributary to them, sporadically in the Adriatic Sea. In Romania these fish may be found along the Black Sea coastline and in the Danube from the Iron Gates to its inflow (863 km).

Specific characteristics: large transversal mouth, protractile, in the shape of an inverted half-moon; the middle of the lower lip is crescent-shaped. The upper lip is continuous while the lower one has a large interruption. Two pairs of barbels reaching almost to the mouth; they are long, flat laterally, and with fringed ends. Normally the beluga sturgeon measures 200–250 cm in length and weighs 100–150 kg, however, it may grow up to 6 m in length and reach over 1000 kg in weight. The adults may range from 30 to 60 years of age; however the older individuals may exceed 100 years.

Characteristics of habitat and reproduction: For their reproduction cycle the beluga sturgeons migrate upstream, sometimes covering great distances from the inflow mouths. The beluga sturgeon has the longest upstream migration. As a consequence, embankments and damming have had a dramatic impact on the natural breeding of this species. Currently, in the Danube, the beluga sturgeons no longer go beyond the dams at the Iron Gates. Two biological forms have been described for the beluga sturgeon: the spring form and the autumn form. The breeding sites are situated in areas with gravel and crevices on the river bed, and with depths of 4–20 m. The males reach sexual maturity at 12–14 years and the females at 14–16 years, when they have a body length of approximately 2 m. After reproduction the males return to sea, where they remain at depths of up to 100 m. The migration of the spawners to the sea is slower and occurs in July-September.

In the Danube, the beluga sturgeon results in natural hybrids with the sterlet, ship sturgeon and Russian sturgeon. In comparison with the genitor species, the hybrids have the tendency to remain in the fresh water environment for longer periods of time. The artificial hybrid *H. huso* x *A. ruthenus* (bester) is reared as a species of economic interest.

The beluga sturgeon is a predatory fish. Its food depends on the season, the adults prefer to feed on other fishes, and juvenile specimens feed on benthic invertebrates.

Acipenser gueldenstaedtii (Brandt, 1833) (Russian Sturgeon)

Distribution: in the Black Sea and the Azov Sea, as well as the Caspian Sea and their tributaries. Apart from the migratory form, there is a fresh water Russian sturgeon, which does not migrate to sea and which lives in the Volga, Ural and the Danube. In Romania, the Russian sturgeon is present along the Black Sea coastline and in the Danube from the Iron Gates to the inflow mouth.

Specific characteristics: the Russian sturgeon has transversal mouth with the lower lip interrupted. Apart from the typical form (short rostrum and barbels insertion reaching almost to the tip of the rostrum rather than the mouth), in the Danube there have been specimens of Russian sturgeon with longer rostrum, in which case the barbels insertion tends to be further away from the tip of the rostrum. The Russian sturgeon has a wide morphologic variability (Antipa, 1909). Thus, Antipa described three rostrum morphs (*typica*, *longirostris* and *acutirostris*) and two varieties of "integument" (naked and with scutes). Other authors consider that these varieties are actually hybrids (Ene and Suciu, 1996). Normally, the Russian sturgeon measures 100–200 cm in length and weighs 20–30 kg; however, it may reach a maximum of 236 cm and 115 kg. The longevity mentioned by several authors is of 33 years (Otel, 2007).

Characteristics of habitat and reproduction: Depending on the migration model, it is assumed that the Russian sturgeon from the Danube has three different intraspecific biological forms: spring, autumn, as well as a sedentary form which does not migrate to sea (Otel, 2007). Depending on the season, the Russian sturgeons live in water with depths ranging from 2 to 100 m. In rivers they live in depths of 2–30 m and swim upstream for the breeding cycles, sometimes to great distances from the inflow mouths. The sexual maturity occurs at ages ranging from 11–13 years in males and 12–16 years in females, when they have reached a body length exceeding one metre. The Russian sturgeon does not breed in consecutive years, but at intervals ranging from 2 to 5 years. The Russian sturgeon give birth to natural hybrids with the sterlet (*Acipenser ruthenus*), stellate sturgeon (*Acipenser stellatus*), ship sturgeon (*Acipenser nudiventris*), beluga sturgeon (*Huso huso*) and common sturgeon (*Acipenser sturio*). Their food consists in various benthic invertebrates and the adults also feed on certain fish species.

Acipenser stellatus (Linnaeus, 1758) (Stellate Sturgeon)

Distribution: in the Black, Caspian and Azov Seas, from where they migrate upstream to their tributaries. The greatest density may be found in the northern area of the Caspian Sea. In the past it was considered to have spread along the entire length of the Danube River and its tributaries; however, currently it does not pass beyond the dam at the Iron Gates.

Specific characteristics: the stellate sturgeon is characterized by a slightly cleaved upper lip, and a lower lip with an interruption in the centre. The rostrum is elongated, narrow and

dorsoventrally flat and its length amounts to 59–65% of the length of the body, which constitutes a particular characteristic, making this species easier to differentiate from the other species of the *Acipenser* genus (Shubina *et al.*, 1989). The body is elongated and fusiform. The mouth is transversal, of medium size. The stellate sturgeon reaches a maximum length of 210 cm and a maximum weight of 68 kg. In the Danube the average weight of the specimens caught is around 6–8 kg. The maximum age mentioned in the literature is of 35 years.

Characteristics of habitat and reproduction: the stellate sturgeon migrates to the sea for feeding and wintering and to the rivers for breeding. The greatest part of the stellate sturgeon's life is spent in the sea. The stellate sturgeon is a species typical for the sea coastal waters and the lower sectors of rivers; however, in contrast with the other sturgeon species, it may also be found in the upper layers of water (Shubina *et al.*, 1989). Adult specimens may be found in pelagic waters, at depths of 10–40 m, and juvenile specimens are to be found closer to the inflow mouths of rivers where there are better places for feeding. For breeding, the stellate sturgeon migrates upstream, sometimes covering great distances from the inflow mouths. The onset of sexual maturity in the Danube is reached at 7–10 years for males and 10–14 years for females, when they reach a body length of 90–120 cm (Manea 1980). The stellate sturgeon does not breed every year. In what regards the breeding migration, there have been mentions of specimens living in the Danube in spring, in the March-April period. There is a secondary period of migration which occurs from August to October, in which case the specimens wintering in the Danube and breed during the April-May period. The preferred sites for breeding are represented by deeper diggings, with depths of 8–10 m, on a rocky substrate, with gravel and ratchel mixed with shell fragments (Shubina *et al.*, 1989). Immediately after breeding the adult individuals return to sea, while the majority of the spawn remains in the river until the autumn. The stellate sturgeon interbreeds with the beluga sturgeon (*Huso huso*), ship sturgeon (*Acipenser nudiventris*), sterlet (*Acipenser ruthenus*) and Russian sturgeon (*Acipenser gueldenstaedti*). In the Black Sea, the spawn feeds on worms, molluscs and crustaceans, while the adults feed on fish and invertebrates (Manea, 1980).

Acipenser ruthenus (Linnaeus, 1758) (Sterlet)

Distribution: the sterlet is an Eurasian species and it is widespread in the rivers that inflow into the Caspian Sea, the Black Sea, the Azov Sea, the Baltic Sea, the White Sea, the Barents Sea and the Kara Sea. This species was introduced through aquaculture in Germany, France, Italy and Belgium. In the Danube the distribution of the species is fragmented.

Specific characteristics: the starlet has a continuous upper lip, and an interrupted lower lip. One of the differences which assist in the differentiation of the sterlet from the other species of the *Acipenser* genus is the fringed barbels (Sokolov and Vasiliev, 1989). The rostrum is elongated, triangular, pointed, slightly raised towards the tip. Antipa (1909) described two varieties of colouring: *alba* (the Latin term for white), with depigmented skin (in which case the eyes may be red) and *erythraea*, in a fire brick red shade. Apart from the typical sterlet shape, with the long and angular rostrum, Antipa (1909) describes a variety with a short rostrum (*Acipenser ruthenus var. brevirostris*). Some authors consider that these forms are in fact distinct genetic groups. Other authors deny the existence of the two forms considering that they are determined by the environment conditions (food) or that they may be the result of hybridization (Sokolov and Vasiliev, 1989). The sterlet may attain a maximum length of 100–125 cm and a maximum weight of 16 kg. The maximum age recorded was of 24 years.

Characteristics of habitat and reproduction: the sterlet is a rheophile fresh water species, preferring the deep areas of great rivers, with strong currents and hard substrate river beds. The sterlet only rarely passes from the Danube to the Black Sea and it is only sporadically found in the Danube Delta. The sterlet is a sedentary species and does not undertake long migrations. The onset of sexual maturity occurs between 3–7 years in males and 5-12 years in females, when the individuals have reached the body length of approximately 40–50 cm. The sterlet interbreeds with the ship sturgeon, beluga sturgeon, stellate sturgeon and most often with the Russian sturgeon. Its food consists of benthic organisms, almost exclusively crustaceans and insect larvae, only occasionally roe and fish spawn.

Hybrids: the interspecific hybridization in vertebrates represents a less frequent phenomenon due to the genetic incompatibility of the parents' genomes (Arnold, 1997) and, generally, these hybrids are not viable. In the case of fish and some amphibians, hybridization occurs more frequently than in the case of other groups of vertebrates. Due to certain characteristics, for instance, polyploidy, sturgeon species hybridize more easily than other fish species (Birstein *et al.*, 1997). It is a well-known fact that sturgeons hybridize in natural conditions, leading, sometimes, to fertile intergeneric or interspecific hybrids. The main cause of hybridization is the overlapping in time and space of the breeding sites. The hybrids are frequently found in the rivers. For instance, it is considered that the sturgeon hybrids from the Volga River represent around 0.02-3.1% of the total number of spawn. In the 1964-1981 period the *A. gueldenstaedtii* x *A. ruthenus* hybrid was the most common (51.3%) among all hybrids encountered in this river.

For an extended period of time, sturgeon hybrids were considered species or subspecies. As a rule, it is considered that the female hybrids from generation F1 obtained through interbreeding of genitor species with the same degree of ploidy are fertile; on the other hand, the hybrids resulted from the interbreeding of genitor species with different degrees of ploidy are sterile (Arefyev, 1997, 1998). Until now the identification of hybrids was carried out based on morphometric characteristics taking into account the fact that they inherit morphological traits characteristic of the species of both genitor species. A relatively high number of morphological descriptions confirm the existence in natural water basins of sturgeon interspecific hybrids (Birstein *et al.*, 1997). Nevertheless, the identification of a certain individual as a hybrid based on morphology is not sufficient and becomes truly complex when three species are involved, as in the case of the hybrid resulted from the interbreeding of the bester (*H. huso* x *A. ruthenus* hybrid) with *A. gueldenstaedtii*. Moreover, the identification of juvenile hybrids based only on morphometric data has been demonstrated to be much more difficult than the identification of adult hybrids. Under these circumstances, both genetic and morphological studies may offer the certain proof of classification as pure species or hybrid.

3. The situation of sturgeon populations in the Lower Danube

From ancient times, sturgeons have had a great economic relevance in the Danube region and they were an important part of the welfare of the local communities from the area. In the old Greek port of Histria the inhabitants were allowed to fish at the Danube inflow mouths and to export salted fish to Greece and Rome, without paying any taxes. Starting with the Middle Age up to the end of the eighteenth century, sturgeons represented an inexhaustible resource of this river (Giurescu 1964). The beluga sturgeon was caught in the

entire Romanian sector of the river, as well as in the middle sector of the Danube, and upstream, up to Bavaria. In the period between the twelfth and the fifteenth centuries, sturgeons were exported from the Danube to Poland (Giurescu, 1964). Starting with the Middle Age more complex tools were used for sturgeon fishing in the Danube. Due to overfishing and poaching, starting with the nineteenth century a decline of the *Acipenseridae* populations in this river has been observed. In the twentieth century, the numbers of sturgeons caught in Romania has decreased dramatically; for instance, in 1994 only 11.5 tons were caught in comparison with approximately 200 tons/year in the 1960s (Bacalbaşa-Dobrovici, 1999). A relevant impact on these species – apart from overfishing – was relegated to the construction of hydroelectric dams on the Danube River.

After the fall of the communist regime, the next decade was characterized by intensive and uncontrolled sturgeon fishing in the Lower Danube, due to a lack of legislation which would regulate the situation of these species of fish (Năvodaru, 1999a; Năvodaru, 1999b; Suciu, 2008). In the period between 2002 and 2005 the sturgeon catches in Romania decreased from 37 tons in 2002, to 11 tons in 2005.

A more thorough analysis of the Danube sturgeon species has shown that *H. huso* is considered to be an extinct species in the Upper Danube, critically endangered in the middle sector of the Danube and vulnerable in the Lower Danube (Hensel & Holcick, 1997). In accordance with other authors this species is extinct in the upper and middle sectors and rare in the Lower Danube (Suciu, 2008). *A. gueldenstaedtii* is considered a critically endangered species in all the sectors of the river and *A. stellatus* is deemed an extinct species in the Upper Danube and the first half of the middle sector of the Danube. In the Lower Danube, the stellate sturgeon population has declined dramatically from a numeric perspective, and the Russian sturgeon populations are on the brink of extinction as a result of an „Allee" effect similar to the one that has led to the disappearance of the *A. sturio* species from the rivers in Western Europe (Suciu, 2008). The arguments brought to support this statement are the decline of catches, the discontinuous structure of the age categories, the lack of natural recruitment or low natural recruitment. In the case of sturgeon populations, the low density of the population is correlated with the negative increase and under these circumstances the populations is condemned to disappearance.

3.1 Impact factors for the sturgeon populations in the Lower Danube

The factors that have led to the dramatic reduction of the sturgeon populations are numerous. Among them, the most important are overfishing, the construction of hydroelectric dams, poaching, pollution and the destruction of breeding habitats.

Since these are species of fish with a very slow growth rate, the sturgeon populations have not been able to cope with this intensive exploitation. The effects of pollution coupled with the construction of dams that have hindered the migration during the breeding cycle have resulted in a considerable decrease in the number of sturgeon populations. Thus, in less than one hundred years these species of fish have been endangered despite their existence for millions of years.

The construction and calling into operation in 1972 of the Iron Gates I Hydroelectric Dam has had a devastating impact on all the sturgeon species that breed within the Danube ecosystem. This dam is situated at 862 km upstream from the inflow mouths of the river into

the Black Sea and it hinders sturgeon migration towards the breeding sites located upstream from the dam. Several hundred years in the past, the Danube sturgeons would reach upstream to Bratislava, Budapest and Vienna. However, nowadays these fish have had to limit their habitat to sea waters and the lower sector of the river (Kiss, 1997).

The sturgeon species that was most affected was the beluga sturgeon. Due to the large sizes of the adults the beluga sturgeons have been unable to reach their breeding sites upstream, despite the existence of the "falling sluices" and "giants" on the dam meant to facilitate the access of fish to breeding sites. At the same time, the area where the dam and reservoir were built used to be an important habitat for spawning for species such as the Russian sturgeon or the beluga sturgeon, since this sector of the river bed is characterized by gravel, fast currents and it is rather deep.

The flooded plains of the Danube have significantly altered when the dams and embankments were built. In the past, the floodable area of the Danube represented 573000 hectares and the delta of the river amounted to 524000 hectares. The Danube Delta has also been embanked, but to a lower extent and the embankments have been halted after the fall of the communist regime in 1989 due to ecological reasons. Thus, the dams and embankments built before that time determined the modification and alteration of entire habitats and trophic networks of which the sturgeon species were an integral part.

A negative impact can also be relegated to the agricultural irrigations and the excavations for building materials from the river bed. The irrigation pumps lead to the destruction of larvae and juvenile fish.

The water pollution is another factor with a significant impact on all the species in the Danube ecosystem. The heavy metal and pesticide pollution is relatively high at the level of the Lower Danube (Oksiyuk et al., 1992) and it affects the biotope comprehensively (Pringle et al., 1993). The pollution level causes relevant problems in the Black Sea as well, where sturgeon populations unfold their trophic cycle. The studies undertaken in this region (Zaitsev, 1993) have shown that the pollution in the area ranges from tens to hundreds of times higher than the one in the Atlantic and Pacific Oceans or in the Mediterranean Sea. The high concentrations of toxins from oil and various industrial wastes modify the hormonal balance of fish, disturb the metabolism and lead to an increase in the number of hermaphrodite fish. Notwithstanding, the breaking up of the former Soviet Union, the main polluter in the Black Sea region, as well as the decrease of the industrialization in the area (including in Romania) and taking more exacting steps to protect the environment, have led to a lower degree of pollution.

Other causes that have resulted in a dramatic decrease in the sturgeon populations were overfishing and poaching. Due to certain characteristics such as large sizes and predictable migration routes, sturgeons are easy to catch. Sturgeon fishing used for meat and caviar represents a profitable business as a result of the expensive prices of these products.

In order to save the sturgeon species in the Danube basin, various steps have been taken aiming to monitor the natural populations, launching efficient programmes for the repopulation and the long term sustainable exploitation of existing reserves. Globally, CITES (Convention of International Trade in Endangered Species of Wild Fauna and Flora) has included the sturgeon species in Annex II in 1998. This initiative was meant to track the regulation of trade with products originating from these species in order to hinder

overexploitation. In order for the sturgeon populations to remain in existence, the decision makers need to bear in mind the fact that the rate of exploitation should not exceed the rate of species regeneration. This ratio is carefully monitored by introducing fishing and exportation quotas. Romania is among the 167 signatories of a convention and together with other countries in the Black Sea region, under the sponsorship of CITES, has founded in 2001 BSSMAG (Black Sea Sturgeon Management Action Group). In 2003, specialists from Romania, Bulgaria, Serbia and Ukraine have established a regional strategy for the protection of these species, focusing on certain aspects such as: maintaining the genetic diversity within the populations, the recovery of the healthy population, able to breed, reconstructing the breeding habitats and developing fisheries to rear sturgeons for consumption as well as roe, leading to market saturation and the elimination of poaching and fishing in the natural environment.

The development of efficient programmes for the management of these extremely valuable natural resources involved various non-governmental organizations. IAD (International Association for Danube Research) adopted an action plan in 2006 for the conservation of *Acipenseridae* species in the Danube River basin („Action Plan for the Conservation of the Sturgeons (*Acipenseridae*) in the Danube River Basin"). In Romania, as a result of the critical situation of the populations, the Ministry of Agriculture, Forestry and Rural Development together with the Ministry of the Environment and Water Husbandry have issued regulations in 2006 meant to strictly forbid the fishing of all sturgeon species on Romanian territory for a period of 10 years. This Order allows the fishing of sturgeons only for aquaculture in order to encourage artificial breeding with the goal to restock the natural habitat with individuals resulted from the crossing of wild genitors.

3.2 European perspectives

Because of the great importance of the sturgeons from both an economical and a scientific point of view there is a growing global interest in studies involving these species. There are many international organizations (World Sturgeon Conservation Society, Wildlife Conservation Society, Natural Resources Defence Council) and research institutes actively involved in the study and conservation of these important natural resources.

In the last years, the release of artificially reproduced fish or the transfer of non-native specimens has become a common practice in conservation programmes intended to reduce the risk of extinction for sturgeon populations. The Elbe, the Weser, the Oder and other German rivers feeding the North Sea and the Baltic Sea once belonged to the habitat of these fish, but the sturgeon population severely declined until extinction. Currently in Germany there is a growing interest for the study of sturgeon species and restocking programs have been initiated in the Baltic Sea. Thus, the beluga sturgeon was introduced in estuaries situated to the east of Rostock city, in the Baltic Sea and more upstream. In these habitats other sturgeon species - *Acipenser gueldenstaedtii* – have also been introduced. Another major concern of biologists in this country is reintroduction of the *Acipenser oxyrhynchus* species in German rivers based on the consideration that the Atlantic sturgeon migrated to the Baltic about 1000 years ago, displacing the European variety, and adopting the river between Germany and Poland as its natural home. Other European countries such as Italy, Spain, Portugal and Hungary are also interested in evaluating the genetic diversity of the existing sturgeon populations and also in saving

these populations from extinction. For example, in Italy initiatives have been undertaken to conserve the *Acipenser naccarii* (Adriatic sturgeon) population in the Po River. Once relatively abundant in the Po River and its estuary, the Adriatic sturgeon underwent a dramatic decline in numbers and became rare by the early twentieth century. In the attempt of saving this population, several measures have been taken such us restocking with artificially reproduced individuals. The restocking actions targeted not only the Po River, but also other rivers from Italy. The Standing Committee of the Bern Convention adopted an international action plan for the restoration of the European sturgeon (*Acipenser sturio*) in November 2007. Countries like France and Germany are directly involved in this international plan and they have already started restoration actions for this sturgeon species.

4. Molecular analyses

The correct identification of sturgeon species is very important for various reasons. Thus, the accurate identification of the species from which the various products, especially caviar, originate is required in order to avoid commercial frauds. The main types of caviar are the beluga, ossetra and sevruga, produced by *H. huso*, *A. gueldenstaedtii* and *A. stellatus*, respectively. These types of caviar differ in taste, price and market availability. As a consequence, there are frequent fraud attempts.

Another aim of the identification of sturgeon species is represented by the protection of these endangered species, this being the first step in the progress of the conservation programs. Taking into consideration the dramatic decline of wild sturgeon populations, that comes dangerously close to disappearance, the practice of repopulating rivers with specimens obtained from artificial reproduction and reared in aquaculture conditions has become increasingly frequent. This phenomenon may have a negative impact on native sturgeon species, by drastically reducing the genetic diversity. In addition, an accurate identification of the specimens to be released in the rivers is also required from a genetic point of view in order to avoid the repopulation with hybrids. It is a well-known fact that sturgeons easily hybridize both in their natural environment and in aquaculture conditions, giving birth to viable and sometimes fertile hybrids. In the case of the sturgeon species in the Danube, the reduction and overlapping of breeding territories have led to a considerably increased chance of interspecific hybridization. Currently, apart from sturgeon species from the Danube, local stugeon fisheries also rear exotic species such as *Acipenser baerii*, as well as their hybrids with *Acipenser gueldenstaedtii*. The majority of fisheries are located near the river or its tributaries; therefore there is an increased risk that these species escape to the Danube River and may threaten the genetic diversity of native species. Ludwig *et al.* (2009) have reported the existence in the Danube of the exotic species *A. baerii*, as well as that of its hybrids with the native species *A. ruthenus.*

The characterization of sturgeons, as a pure species or hybrid is difficult to achieve based exclusively on morphometric criteria, especially in the juvenile stage. Taking into account the previously mentioned aspects, it is evident the importance of the characterization of Danube sturgeon species both on morphological and meristic criteria, as well as from a genetic point of view.

The development of DNA markers has had a revolutionary impact on genetics in generally, and on fish genetics implicitly, as well as on its application in aquaculture. The application of molecular markers in fish has enabled the recording of some rapid progresses regarding the studies of genetic variability and inbreeding, determining the paternity, species identification, building genetic linkage maps for aquaculture species, by identifying the loci correlated to certain quantitative traits (QTL - Quantitave Trait Loci) for marker-assisted selection.

In order to perform some molecular analyses, we have collected biological samples from the following Danube sturgeons: *A. stellatus*, *A. gueldenstaedtii*, *A. ruthenus* and *H. huso*. For some of these analyses we have included control samples originating from the aquaculture species: *A. baerii*, *A. gueldenstaedtii* and the interspecific hybrid *A. baerii* X *A. gueldenstaedtii*.

The samples were represented by fin fragments and their collection did not require the death of the sampled individuals. This particular aspect is extremely important in the case of species which are on the brink of extinction and are included in conservation programs. The fin fragments were fixed in 96% ethanol before DNA isolation. The total DNA was isolated by using a specific extraction method with phenol-chloroform-isoamyl alcohol (Taggart et al., 1991) or the the Chelex extraction method (Estoup et al., 1996).

In order to identify the Danube sturgeon species there are various techniques that may be employed.

4.1 PCR-RFLP (Polymerase Chain Reaction - Restriction Fragment Length Polymorphism)

This technique is based on the amplification by PCR of a specific DNA region which comprises one or more polymorphic sites for various restriction enzymes. Along with the increase of the number of "universal" primers available in the literature, different DNA regions which are relatively conserved (for instance, mtDNA) can be targeted. Furthermore, PCR products can be digested with restriction enzymes and visualized by ethidium bromide staining due to the increased quantity of DNA resulted from the amplification reaction. Since the size difference between restriction fragments is usually noticeable, these fragments may be observed relatively easily by electrophoresis.

The literature on sturgeons mentions a relevant number of studies based on RFLP-type markers. These markers were initially used as a focus in two directions: in order to establish the genetic diversity of sturgeon populations with a distinct geographic distribution (Miracle & Campton, 1995; Szalanski et al., 2001; Waldman et al., 2002) and in order to establish the usefulness of these markers for recovering the phylogeny of the *Acipenseridae* class (Brown et al., 1996). In recent years PCR-RFLP was proposed as a molecular method for the identification of sturgeon species and implicitly for the determination of the origin of the caviar on the market. Wolf et al. (1999) identified various species-specific restriction profiles at the level of a 462bp fragment from the mitochondrial gene for cytochrome b in the cases of 10 species from the *Acipenser* and *Huso* genera. Ludwig *et al.* (2002) have extended the study to 22 sturgeon species, by investigating the restriction profiles resulting from the action of five enzymes on a 1121bp fragment representing the entire gene for cytochrome b. Advantages:

i.   The simplicity of the protocol (a single pair of primers is necessary).
ii. The technique is applicable for the accurate identification of all Danube sturgeon species for breeding, propagation, cryopreservation in order to exclude the possibility of misidentification.

Disadvantages: the main inconvenient of RFLP is the fact that it is possible to identify a relatively low degree of polymorphism. In addition, it requires the knowledge of a particular sequence, which makes difficult to find to find new markers in the species for which the known molecular data is quite reduced. The identification of species through PCR-RFLP is efficient for other vertebrate species, including fish, but it has been proven to be indecisive in the case of *Acipenseridae*.

Thus, it is proven to be very difficult to identify specific substitutions with diagnostic role for species that are very close genetically and evolutionary, as is the case of the *A. gueldenstaedtii/A. persicus* species and of the three species of the *Scaphirhynchus* genus. On the other hand, the analysis of restriction fragments length polymorphisms of mitochondrial DNA has the disadvantage that mtDNA is inherited exclusively through the maternal lineage. This may lead to erroneous interpretations, as in the case of hybrid identification and/or of ancient introgression, since both phenomena may be found in the case of sturgeons and are reported in the literature (Arefyev, 1997; Ludwig et al., 2003; Tranah et al., 2004).

In order to identify the Danube sturgeon species, we have amplified by PCR a 462bp fragment on the level of the RNA_t^{Glu}/cytochome b mitochondrial region. The fragment was subsequently subjected to restriction endonuclease digestion (in accordance with the method proposed by Wolf *et al.*, 1999), thus obtaining species-specific restriction profiles.

In order to amplify the RNA_t^{Glu}/cyt b fragment we used a single pair of primers: F (AAAAACCACCGTTGTTATTCAACTA) (Brugner & Hubner, 1998) and R (GCCCCTCAGAATGATATTTGTCCTCA) (Kocher *et al.*, 1989). These primers are annealing to conserved regions of mtDNA and they allow the amplification of the fragment of interest for all analyzed sturgeon species.

In order to identify the sites with a role in the diagnosis and selection of enzymes that allow the interspecific discrimination, the fragments obtained were sequenced by the "dye terminator" method (using *Applied Biosystems* reagents and equipment) and they were aligned with Clustal X 2.0.9 (Larkin *et al.*, 2007) (Figure 1).

Thus, for the restriction reaction we selected the *SspI*, *RsaI* and *Tru9I* enzymes. The digestion of the 462bp fragment with the three restriction enzymes followed by the 3.5% agarose gel electrophoresis resulted in species-specific restriction profiles (Table 1). Fragments which are smaller than 50bp could not be visualized by gel electrophoresis.

The calculated RFLP profiles as a result of the sequence analysis were confirmed experimentally, given that after the digestion with the three enzymes we obtained the species-specific profiles presented in figures 2, 3, 4.

In the case of the hybrids analyzed, it was demonstrated that they present a nucleotide sequence identical with that of the maternal species, *A. baerii*. This proves that such methods are limited in their utility for the identification of pure sturgeon species. In the case of hybrids this method is limited to the identification of their maternal origin. Although
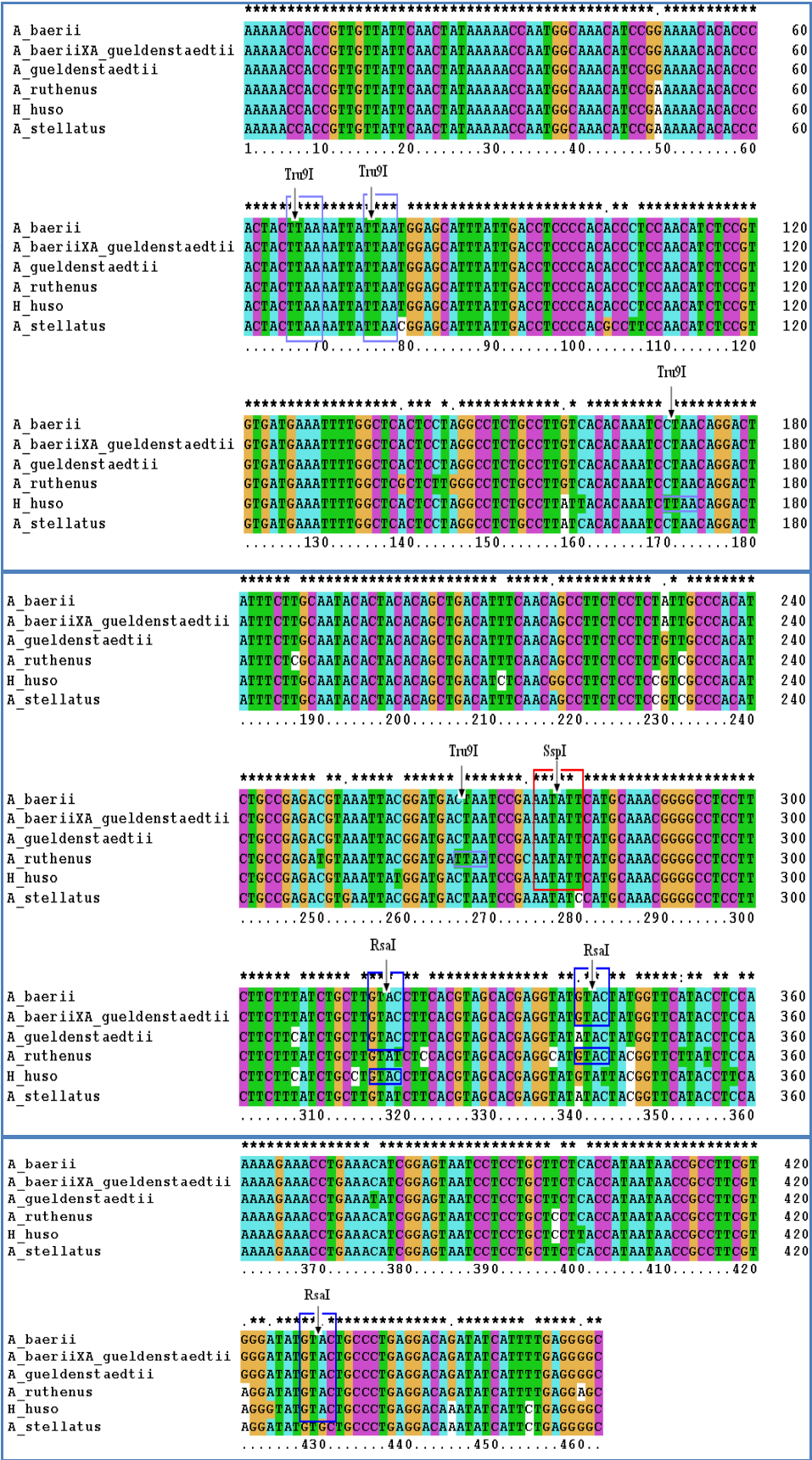
Fig. 1. Alignment of the RNAt^{Glu}/cytochrome b fragment in the analyzed sturgeon species.

mitochondrial markers are frequently used for identifying species, we must take into account the exclusively maternal inheritance of mtDNA, given that this characteristic may lead to erroneous interpretations in the identification of hybrids and/or introgression.

	H. huso	*A. stellatus*	*A. ruthenus*	*A. gueldenstaedtii*	*A. baerii*
SspI	277;185	462	277;185	277; 185	277;185
RsaI	317;112;33	462	341;88;33	317;112;33	317;88;33;24
Tru9I	292;95;66;9	387;66;9	196;191;66;9	387;66;9	387;66;9

Table 1. Restriction polymorphisms of the RNAt^{Glu}/cytochrome b fragment in sturgeons (fragments length is represented in base pairs).
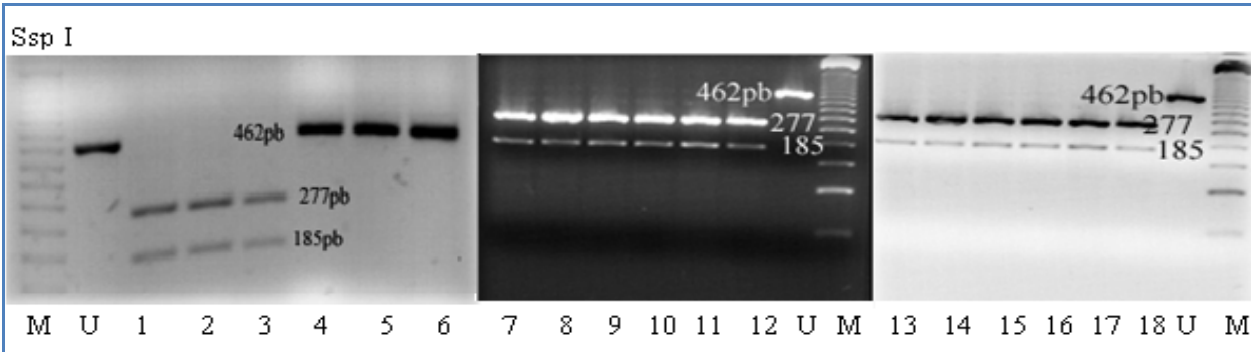

Fig. 2. The electrophoretic profile obtained after the digestion of the RNA$_t$^{Glu}/cytochrome b fragment with *SspI*. M – molecular size ladder 50bp; U–uncut amplicon; 1,2,3–*H. huso*; 4, 5,6–*A. stellatus*; 7,8,9–*A. ruthenus*; 10,11,12–*A. gueldenstaedtii*; 13,14–*A. baerii*X*A. gueldenstaedtii* (aquaculture); 15,16–*A. gueldenstaedtii* (aquaculture); 17,18-*A. baerii* (aquaculture).
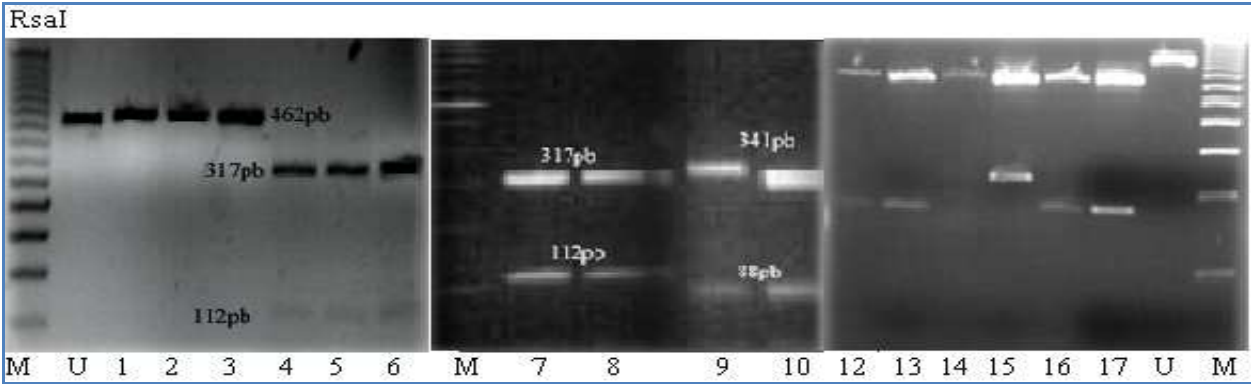

Fig. 3. The electrophoretic profile obtained after the digestion of the RNA$_t$^{Glu}/cytochrome b fragment with *RsaI*. M - molecular size ladder 50bp; U - uncut amplicon; 1,2, 3–*A. stellatus*; 4,5,6 –*H. huso*; 7, 8 –*A. gueldenstaedtii*; 9, 10 –*A. ruthenus*; 11, 12 –*A. baerii* X *A. gueldenstaedtii* (aquaculture); 13, 14 –*A. gueldenstaedtii* (aquaculture); 15, 16 - *A. baerii* (aquaculture).
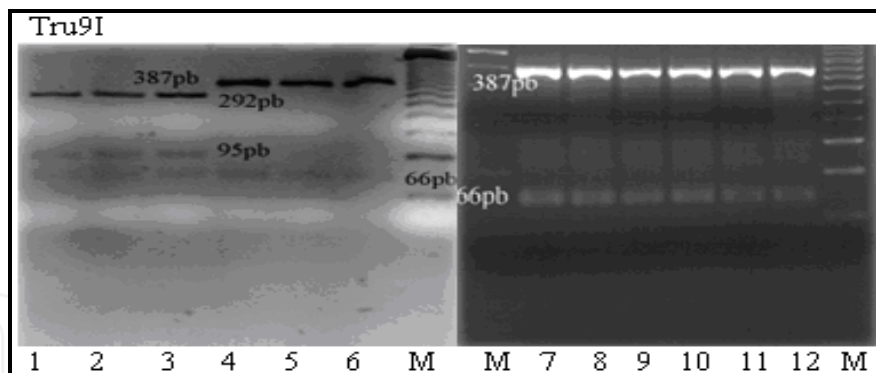
Fig. 4. The electrophoretic profile obtained after the digestion of the RNA$_t$^{Glu}/cytochrome b fragment with *RsaI*. M - molecular size ladder 50bp; 1,2, 3–*A. stellatus*; 4,5,6–*H. huso*; 7, 8 –*A. gueldenstaedtii*; 9,10–*A. baeriiXA. gueldenstaedtii* (aquaculture);11,12–*A. baerii* (aquaculture).

4.2 Amplified Fragment Length Polymorphism (AFLP)

In the case of the amplified fragment length polymorphism technique, the DNA chain is subjected to digestion by two restriction enzymes, for instance, *EcoRI* and *MseI*. *EcoRI* has a 6bp recognition site and rarely cleaves, and *MseI* has a 4bp recognition site and frequently cleaves. Specific adaptors are bound to the resulting restriction fragments and, subsequently, only a part of the restriction fragments are amplified by PCR. The selection of these fragments is done by using primers which are complementary to the adaptor sequence, the restriction site and several nucleotides from the restriction fragment. The amplified fragments are separated by polyacrylamide gels electrophoresis and visualized through autoradiography or fluorescent labeling.

The markers detected through the AFLP technique present a multiple band profile profile for each individual and in some cases researchers have observed that this profile is specific to one of the genders or a certain species. In the case of sturgeons some studies have been undertaken by Congiu *et al.* (2001, 2002) in order to identify the hybrids that appear in the natural environment or as a result of selective crossbreeding in aquaculture. Apparently, the profile of the bands allows the identification of hybrids when the profiles of each genitor species are used as reference.

4.3 Microsatellites analysis

Microsatellites (also called VNTR - Variable Number of Tandem Repeats) are short repetitive sequences, of 2-9bp, dispersed in the entire genome and they have a high degree of polymorphism. These markers are numerous in all vertebrate species, and in fish they appear at approximately every 10 kB (Wright, 1993).

Due to some of their characteristics, such us the relatively reduced size, easiness with which they could amplified by PCR, the co-dominant inheritance mode and the high degree of polymorphism, microsatellites are very useful markers. They may be used in numerous studies from varied fields of molecular biology, including molecular epidemiology, population genetics and genetic mapping. In the aquaculture and fish genetics studies, microsatellites are used for the genetic characterization of stocks, specimen selection for breeding, linkage map building, mapping and gene identification for QTL and various applications in the assisted breeding programs.

Microsatellite analysis results in individual genetic profiling (genetic fingerprinting). It may be used to establish the interrelations between various individuals and assess their allele frequency within the populations. Taking into account the molecular methods currently available, it is possible to assess length polymorphisms in a great number of individuals for intra- and inter-population genetic analyses. Certain microsatellites possess a high number of allele per locus and they are, therefore, appropriate for the identification of genitors, and their descendents, respectively, in mixed populations. Other microsatellites possess a lower number of alleles and are recommended in the genetic and phylogeny studies of populations (O'Connell & Wright, 1997; Estoup & Angers, 1998).

Currently, most laboratories analyzing microsatellites use the genotyping by PCR amplification with primers that bind to the sequences flanking the repetitive motifs. The resulting amplification products are separated depending on size by polyacrylamide gel electrophoresis or capillary electrophoresis in automated genetic analyzers.

In the case of sturgeons, the studies based on microsatellite markers analysis were initiated for the North American species. Currently, each of these species was studied from the point of view of genetic diversity, despite the difficulties in collecting the biological samples. These difficulties are reflected in the reduced number of analyzed specimens. Taking into account the fact that microsatellites contain important genetic information for the comparison of sturgeon populations, the research directions were especially aimed at the development of disomic microsatellites in the North American sturgeon species and paddlefish (May *et al.*, 1997; McQuown *et al.*, 2000; King *et al.*, 2001; Pyatskowit *et al.*, 2001; Heist *et al.*, 2002; Henderson- Arzapalo & King, 2002; Welsh *et al.*, 2003; Welsh & May, 2006). The identification and characterization of new microsatellite loci in sturgeons is difficult due to the polyploidy of these fish. Many potential microsatellites were eliminated from research studies since they were polysomic and they tended to complicate the interpretation of the inheritance mode and the intra- and inter- population genetic variation. Nevertheless, for the North American species, researchers have developed a set of disomic loci and microsatellites have begun to be used increasingly in population genetics studies. Researchers have identified various levels of genetic diversity, population structures and sturgeon stocks using different molecular markers in different species.

Another application of microsatellites in sturgeons was focusing on the use of these markers for species identification. Thus, these molecular markers with Mendelian inheritance represent a viable solution for the detection of hybridization. They also allow the characterization of individuals as pertaining to pure species or hybrids. Microsatellites can be very useful in the identification of sturgeon hybrids, by taking into consideration the fact that the alleles of an individual represent a combination of the parental alleles.

In the case of the species that spawn in the Danube, we tested various microsatellite loci. Finally, we selected eight of the microsatellite loci tested (LS19, LS34, LS39, LS54 - May et al., 1997; Aox27 – King et al., 2001; AoxD234 – Henderson – Arzapalo & King, 2002; AnacE4, AnacC11 – Forlani et al., 2008). These eight microsatellite loci have demonstrated a very good amplification, result repeatability and interspecific polymorphism in all of the four investigated species.

Allele frequencies were calculated and private alleles were identified (alleles that appear only in one of the analyzed species) given that they are considered diagnostic alleles. These

Locus	Alleles size (bp)	*A. stellatus*	*H. huso*	*A. gueldenstaedtii*	*A. ruthenus*
LS19	133	0.5238	0.0238	0.1538	0
	136	0.0952	0.0714	0.359	0.5
	139	0.1905	0.0952	0.0641	0.4375
	140	0	0	**0.0128**	0
	142	0.1667	0	0.1282	0.0625
	145	**0.0238**	0	0	0
	148	0	0.0714	0.1923	0
	151	0	**0.0476**	0	0
	154	0	**0.0952**	0	0
	157	0	0.119	0.0897	0
	160	0	**0.4286**	0	0
	163	0	**0.0476**	0	0
LS34	**139**	0	0	0	**0.0313**
	142	0	0.9762	1	0
	145	0	0	0	**0.9688**
	148	1	0.0238	0	0
Aox27	**114**	0	0	**0.4605**	0
	118	0	0	0	**1**
	122	0	**0.1905**	0	0
	126	0.0476	0.1429	0.0132	0
	130	0.0714	0.4048	0	0
	134	0.1905	0.2619	0.2895	0
	138	0.3333	0	0.2105	0
	142	**0.1429**	0	0	0
	146	**0.1905**	0	0	0
	150	0	0	**0.0263**	0
LS54	**140**	**0.0476**	0	0	0
	152	0	0	0	**0.9375**
	160	0.1905	0	0	0.0625
	164	**0.0952**	0	0	0
	172	**0.0476**	0	0	0
	176	**0.0952**	0	0	0
	184	**0.2143**	0	0	0
	188	**0.2619**	0	0	0
	192	**0.0476**	0	0	0
	196	0	0	**0.2308**	0
	204	0	0	**0.2051**	0
	208	0	0	**0.2308**	0
	212	0	0	**0.3077**	0
	215	0	0	**0.0128**	0
	220	0	**0.0952**	0	0
	232	0	**0.0714**	0	0
	236	0	**0.2619**	0	0
	240	0	**0.0714**	0	0
	244	0	0.1429	0.0128	0
	248	0	**0.0952**	0	0
	252	0	**0.2619**	0	0

LS39	**96**	**1**	0	0	0
	102	0	0	0	**0.0625**
	105	0	0.2143	0.7368	0.625
	108	0	0.6667	0.2632	0.3125
	111	0	**0.0714**	0	0
	114	0	**0.0476**	0	0
AnacE4	326	0	0	0.1795	0.4688
	328	0	0	0.4744	0.4375
	332	0	0.119	0	0.0938
	334	0	0.5952	0	0
	336	0	0.0238	0.0385	0
	338	0	0.0476	0.0769	0
	346	**0.1905**	0	0	0
	348	**0.2143**	0	0	0
	350	0.1905	0	0.0128	0
	352	0.1667	0	0.0128	0
	354	**0.1905**	0	0	0
	358	0.0476	0.0714	0	0
	360	0	0.1429	0.2051	0
AoxD234	**195**	0	**0.0238**	0	0
	199	0	**0.0238**	0	0
	203	**0.1429**	0	0	0
	207	0.0952	0.0476	0.2361	0
	211	0	**0.0238**	0	0
	215	0	0.2381	0.3056	0
	219	0	0.0238	0	0.1563
	223	**0.1905**	0	0	0
	227	0.1429	0.2381	0.1667	0
	231	0	0	**0.0278**	0
	235	**0.2857**	0	0	0
	239	0	**0.0476**	0	0
	243	0	**0.0714**	0	0
	247	0.1429	0.0952	0.0833	0
	251	0	0.1667	0.0833	0.0938
	255	0	0	0.0417	0.25
	259	0	0	0	0.2813
	271	0	0	0.0556	0.2188
AnacC11	**145**	0	**0.4048**	0	0
	153	**0.125**	0	0	0
	161	0	0	0	**0.25**
	165	0.1	0.1667	0.027	0
	169	0	0	0.1892	0.2188
	173	0.225	0.4286	0	0
	177	0.425	0	0.5676	0.2188
	185	0.1	0	0	0.25
	189	0	0	**0.2162**	0
	193	**0.025**	0	0	0
	201	0	0	0	**0.0625**

Table 2. Allele frequencies calculated with the Genetix software (Belkhir *et al.*, 2002) for the Danube sturgeon populations. The allele frequencies in bold indicate the potential diagnostic alleles.

diagnostic alleles are distributed in a wide frequency range, from 0.01 to 1 (fixed alleles for that particular population) (Table 2). Taking into account the co-dominant inheritance mode, these private alleles may be used as markers for the identification of hybrids. This method assumes the analysis of a significant number of specimens from the parental species in order to obtain the confidence of an accurate diagnostic.

4.4 Sequencing

The analysis of nucleotide sequences and of the existing degree of variation at their level allows the identification of sturgeon species, subspecies or populations. The sequences recorded in the various data bases such as GenBank or EMBL, for a significant number of markers from various species, are available for researchers for comparison and analysis. As mentioned earlier, sequencing also represents a validation method of the results obtained through various methods (for instance, RFLP or PCR amplification with species-specific primers). Sequencing constitutes the most precise diagnostic method but it also has an important disadvantage by being, for the moment, an expensive technique, which takes a lot of time and, as a consequence, is not suitable for analyzing numerous samples. Currently, due to the development of the Next Generation Sequencing Systems, this technique has become more accessible and it allows the analysis of an extended set of markers for a significant number of species and/or specimens.

It is preferable that, before the development of mitochondrial or nuclear markers for the identification of sturgeon species, to take into consideration the phylogenetic relations established at the level of this group. The establishment of phylogenetic relations in sturgeons is important in order to understand the genetics of these species with an ancient origin, since there are considered genuine "living fossils". Furthermore, taking into account the fact that many of the sturgeon species are on the brink of extinction, it is important to establish the phylogenetic relations in this group of fish because there is a risk that in the future such studies would be focused only on a reduced number of species /specimens from museum collections.

One interesting aspect to consider is the fact that the phylogenetic relations within the *Acipenseriformes* order, as established based on molecular data, present some differences comparing to those established as a result of morphological analyses.

For the phylogenetic classification of the sturgeon species belonging to the *Acipenseriformes* order, which live in the Black Sea and spawn in the Lower Danube, we have sequenced the cytochrome b (1141bp), cytochrome oxidase I (678bp) and cytochrome oxidase II (667bp) mitochondrial markers. In this phylogenetic analysis we have included, apart from our own sequences, 26 sequences already recorded in GenBank (25 from sturgeon species, and as outgroup we have selected the *Polypterus ornatipinnis* species, as a representative of the closest taxon to sturgeons – the *Polypteriformes* order). The phylogenetic tree was built by using the Neighbor-Joining (NJ) method implemented in the MEGA4 software (Tamura *et al.*, 2007), based on the Tamura-Nei plus gamma evolutionary model established with ModelTest (Posada & Crandall, 1998) (Figure 5).

The tree obtained shows a separation in the *Acipenseriformes* order into two monophyletic clades: *Polyodontidae* (represented by *Polyodon spathula* and *Psephurus gladius*) and *Acipenseridae*. The American Atlantic sturgeon (*A. oxyrhinchus*) and the common European

Atlantic sturgeon (*A. sturio*) form a monophyletic group and occupy a basal position in the *Acipenseridae* family. This conclusion confirms other molecular phylogeny studies (Krieger *et al.*, 2008), but it is in contradiction with morphometric data, which places in a basal position the group formed by the species of the *Scaphirhynchus* genus (Mayden & Kuhajda, 1996; Birstein & DeSalle, 1998).
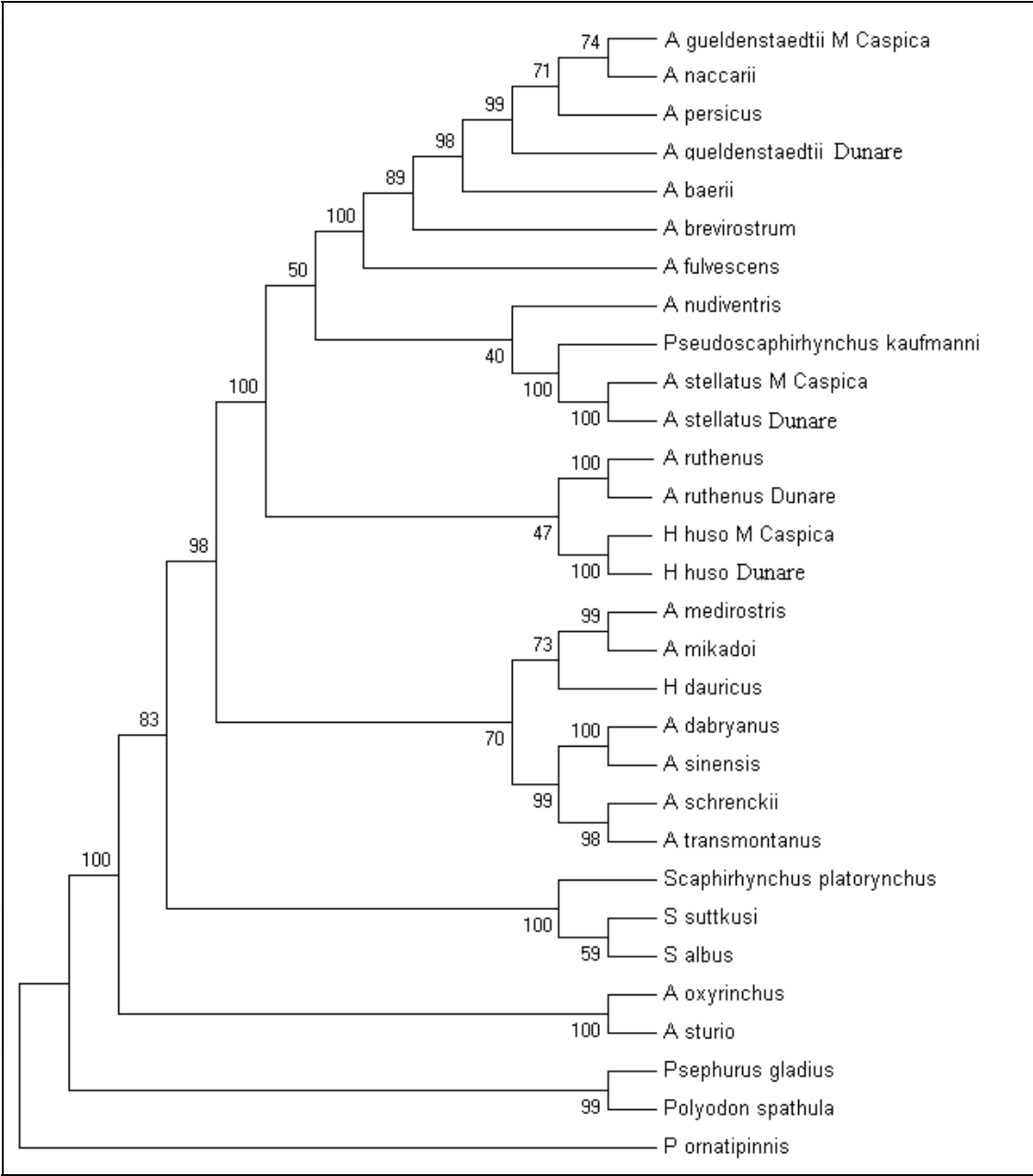

Fig. 5. Phylogenetic trees built through the NJ method based on the cytochrome b + cox I + cox II (2486bp) sequences.

Within the trees obtained, in the Acipenseridae family, two major clades were observed: the Atlantic clade (*A. ruthenus, A. nudiventris, A. stellatus, A. fulvescens, A. brevirostrum, A. baerii, A. persicus, A. gueldenstaedtii, A. naccarii and H. huso*) and the Pacific clade (*A. sinensis, A. dabryanus, A. medirostris, A. mikadoi, A. transmontanus, A. schrenckii and H. dauricus*). The

Pontic-Caspian species analyzed, among which we included the sturgeon species spawning in the Lower Danube, were distributed in the Atlantic clade.

The NJ analyses based on concatened mitochondrial sequences have shown that *H. huso* and *A. ruthenus* form a cluster, as directly related species. This classification is surprising, given the fact that *H. huso* and *A. ruthenus* present very different traits from a morphological and ecological point of view. The hypothesis that *H. huso* and *A. ruthenus* are closely related is supported by the easiness with which they hybridize, since their hybrids are viable and fertile (Birstein et al., 1997). The results we have obtained may be explained by an introgression of the mitochondrial genome from *H. huso* to *A. ruthenus* which took place in the remote past. It is possible that the analyzed specimens, although not hybrids, could have derived from a mitochondrial line of hybrid origins. Recently, the "bottleneck" phenomenon has been observed within the populations. This phenomenon encourages the fixation of a certain mitochondrial line or the introgression phenomenon (for instance a haplotype from *A. ruthenus* may become dominant in *H. huso* or the reverse may be true).

Under these circumstances, the interrelation between *H. huso* and *A. ruthenus* is artificial and it results from the history of the genes rather than from the history of the species. Similar results which place *H. huso* and *A. ruthenus* in a monophyletic group have also been obtained by Birstein & DeSalle (1998); Birstein et al., (2000) and Krieger et al., (2008).

The two species of the *Huso* genus are distributed among the species of the *Acipenser* genus, which means that the *Huso* genus is not monophyletic and it does not form a separate taxonomic unit. *A. stellatus* forms a monophyletic group with *P. kaufmanni*, and *A. gueldenstaedtii* with the Euroasian species *A. baerii*, *A. persicus* and *A. naccarii*.

4.5 DNA barcoding

"DNA barcoding" is a technique used in order to characterize species using short and standardized DNA sequences. The process involve the organization of a reference collection, obtaining DNA sequences and introducing them into a data base and including in a standardized analysis. The best candidates for the identification of animal species are mitochondrial genes which encode for proteins, these being preferred to those encoding for rRNA16S and rRNA12S. These genes present more interspecific variations and as a result they are able to differentiate even between closely related species. Moreover, the analysis of genes encoding for proteins is facilitated by the fact that, generally, they do not present mutations of the insertion and/or deletion type which occur frequently in the mitochondrial genes for rRNA. One region of 650bp from the 5' end of the gene for CO I was proposed as a potential "barcode" for species identification, taking into consideration the following advantages:

a. The easiness of amplification, using a reduced number of primers for different taxa;
b. The efficiency in discriminating among closely related species in a wide variety of invertebrates and vertebrates.

Once a data base containing the CO I sequences from various species was created and the nucleotides with a key role have been identified, the short species-specific oligonucleotides may be synthesized and placed on a DNA chip (similarly to a "microarray" analysis). In the case of sturgeons the implementation of such a technique may be extremely useful for the identification of species, especially in correlation with the accurate labeling of caviar origin.

5. Conclusions

The accurate identification of sturgeon species and hybrids is very important both from a commercial point of view as well as from the perspective of protecting these extremely valuable species. The decision to initiate repopulation programs in order to recover the natural sturgeon populations must take into account not only the social and economic aspects, but also the assessment of genetic diversity and the correct diagnostic of the species involved.

The diagnostic of species based on mitochondrial markers through PCR-RFLP or sequencing may be applied only in the case of pure species. Hybrids cannot be identified through the analyses of these markers, since they display restriction profiles and mitochondrial haplotypes similar to those of the maternal genitor species. This fact is not surprising due to the exclusively maternal inheritance trait of mtDNA. The analysis of mitochondrial markers is useful in determining the origin of the maternal species involved in the hybridization. For the identification of hybrids and of the genitor species involved in hybridization nuclear markers as microsatellites seems more appropriate. An accurate diagnostic of the sturgeon species and hybrids is very important if we take into consideration their fragile status and such a diagnostic can result only from both morphological and molecular data.

6. Aknowledgements

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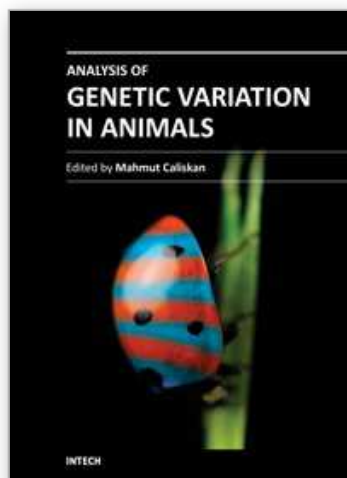
Analysis of Genetic Variation in Animals

Edited by Prof. Mahmut Caliskan

Analysis of Genetic Variation in Animals includes chapters revealing the magnitude of genetic variation existing in animal populations. The genetic diversity between and within populations displayed by molecular markers receive extensive interest due to the usefulness of this information in breeding and conservation programs. In this concept molecular markers give valuable information. The increasing availability of PCR-based molecular markers allows the detailed analyses and evaluation of genetic diversity in animals and also, the detection of genes influencing economically important traits. The purpose of the book is to provide a glimpse into the dynamic process of genetic variation in animals by presenting the thoughts of scientists who are engaged in the generation of new idea and techniques employed for the assessment of genetic diversity, often from very different perspectives. The book should prove useful to students, researchers, and experts in the area of conservation biology, genetic diversity, and molecular biology.

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