NTNU

Norwegian University of
Science and Technology

Conservation of genetic diversity in populations of stellate sturgeon *(Acipenser stellatus)* of the NW Black Sea and Lower Danube River

Daniela Nicoleta Holostenco

Marine Coastal Development
Submission date: May 2011
Supervisor: Jarle Mork, IBI
Co-supervisor: Dalia Florentina Onara, Danube Delta National Institute, Romania
Marieta Costache, University of Bucharest, Faculty of Biology, Romania

Norwegian University of Science and Technology
Department of Biology

Abstract

The stellate sturgeons as well as the other sturgeon populations inhabiting the Lower Danube River system (LDR) and the NW part of the Black Sea have experienced a severe decline due to heavy fishery, river regulation and water pollution. Traditionally, sturgeons have represented a great economic value for the countries along the Danube, being fished mainly for their eggs, the "black caviar". The human population along the Danube River took advantage of the anadromous behaviour of these fishes and over decades of overfishing they depleted the stocks. In 2006 the Romanian government banned the commercial fishing of sturgeons for a period of 10 years and started a Supportive Stocking Programme of Danube with farmed juveniles originating from wild parents.

The present study focuses on whether there are genetically isolated subpopulations among the adult cohorts of stellate sturgeons migrating into the LDR in the year 2010, and whether the genetic characteristics of YOY samples in the same year supports the existence of such subpopulations.

Samples from fall and spring migrants and from YOY stellate sturgeons naturally born in the river in 2010 were genetically analyzed for mtDNA *cyt b* and *D-loop* regions as well as a panel of nuclear microsatellites, and revealed considerable individual variability. Hardy-Weinberg conformity tests revealed heterozygote deficit at 3 out of 4 microsatellite loci. Based on the genic differentiation at the microsatellite loci and mtDNA and cluster analyses, a genetically distinct group were demonstrated among the 2010 YOY samples, pointing to the existence of a previously unknown population substructure within the stellate sturgeons of The Lower Danube River. This was supported by biological characteristics of the YOY samples.

Contents

Introduction **1**
Rationale of the Study 2
The hypothesis of the study 2
Objective of the study 2
Background information 3
1. Distribution of Acipenser stellatus Pallas 3
2. Biology of the species 4
3. Life history and ecology 4
4. Hybridisation 8
Stock fluctuations 8
Supportive stocking programme 12
Baseline genetic information 14
Materials and methods **16**
Sample collection 16
Primers and analytical software used 20
1. Mitochondrial DNA 20
PCR 20
RFLP 21
2. Microsatellites DNA 23
PCR 24
3. Statistical analyses 24
Results **25**
1. Mitochondrial DNA 25
2. Microsatellites 27
3. Results from the statistical analysis 29
Hardy-Weinberg conformity 30
Linkage disequilibrium (LD) 30
Genic differentiation 31
Testing for null alleles at microsatellite loci 31
Haplotype variability at mtDNA 32

Cluster analysis and dendrogram construction using the DISPAN programme suite 33
4. Tests of working hypotheses 1-3 34
Discussion 36
Study case - YOY 37
Conclusions 41
Acknowledgments 43
Bibliography 45
Appendix 50

Introduction

From ancient times, sturgeons have had great economic importance in the Danube River region, forming basis for a significant commercial fishery. Historically five sturgeon species have been migrating from the Black Sea and up the Danube River for spawning: beluga (*Huso huso*), Russian sturgeon (*Acipenser gueldenstaedti*), stellate sturgeon (*A. stellatus*), ship sturgeon (*A. nudiventris*), and perhaps European Atlantic sturgeon (*A. sturio*) (Bacalbaşa-Dobrovici 1997).

Sturgeons have been harvested on a commercial scale since the 4th and 5th centuries, and from the Middle Ages, sophisticated gear was developed taking advantage of the anadromous movements of these fish (Knight, Kristensen et al. 2009). During the 20th Century as a result of dam construction, pollution, habitat changes and over-fishing, all Danube sturgeons have declined. Commercial catches declined considerably and all species are considered endangered (Ferguson, Suciu et al. 2000). Due to the threats mentioned above, these anadromous sturgeons are now critically endangered or even extinct in the upper and middle Danube reaches (Hensel and Holčík 1997).

In the Romanian part of the lower Danube, only three anadromous species still occur: *Huso huso, A. gueldenstaedti* and *A. stellatus.* These populations are affected greatly by the dams and other river installations (Bacalbaşa-Dobrovici 1997).

The Danube river system has long been subjected to anthropogenic influence from a number of sources, altering the entire ecosystem. The fish community and sturgeon species in particular have been greatly impacted by these changes (Knight, Kristensen et al. 2009). The hydroelectric dams Iron Gate I & II at river kilometres 942 and 863, constructed in 1970 and 1984 respectively, divide the lower and mid Danube. As a result, the historic spawning migration of Danube sturgeon, which was documented to extend as far upstream as Vienna (Bacalbaşa-Dobrovici 1997), has been halved (Knight, Kristensen et al. 2009)

Despite the risks connected within complete knowledge of the genetic structure (Ferguson, Suciu et al. 2000), (Suciu, Prodohl et al. 2001), low to medium level supportive stocking programmes were initiated in 2005 for stellate and Russian sturgeons of the region to

compensate the effects of the intensive fishing in Romania during 1900 – 2000, prior to implementing of CITES regulations (Paraschiv, Suciu et al. 2006).

In 2006, all sturgeon fishing was banned for ten years by the Romanian government, with the exception of a low number of live individuals to be used for a supportive stocking programme, which are to be returned live to the Danube afterward (Romanian Sturgeons and CITES website-fishing moratorium).

Today, it is widely accepted that a sustainable use of fish resources must include comprehensive monitoring of the state of populations (stocks) involved and a toolbox of management measures that can be used to regulate the exploitation (Hallerman 2003).

Rationale of the Study

In the case of the stellate sturgeon populations in the NW Black Sea and lower Danube River (LDR), which are the focus of the present project, information on general biology (demography, behaviour, migrations, growth and maturation) and ecology (interactions with other species and the environment), as well as a baseline genetic status of the populations involved are critically required to secure the conservation of genetic diversity within and between populations for future (Hallerman 2003). Furthermore, the genetic population structure (the real reproductive units) must be known; otherwise most of the common management tools and regulations may be without effect or worse - have unwanted effects.

The hypothesis of the study

The working hypothesis of this project is that mtDNA and nuclear markers can be used to detect and distinguish between reproductive units / sub-populations of the LDR and NW Black Sea stellate sturgeons.

Objective of the study

The aim of the present study was to examine whether *D-loop* and *cytochrome b* regions of the mtDNA and the LS-19, LS-34, LS-54, LS-57, LS-68, Aox-23 and Aox-45 nuclear microsatellites could be used to distinguish sub-populations within the cohorts of adult stellate sturgeons migrating into the LDR as well as in the YOY (Young-of-the-Year) stellate sturgeons born in the LDR during 2010.

Background information

1. Distribution of Acipenser stellatus Pallas, 1771 (Stellate Sturgeon)

Acipenser stellatus inhabits the Aegean, Black, Azov and Caspian Seas with the largest density in the Northern Caspian Sea (Fig. 1).

Fig. 1. *Acipenser stellatus* Pallas, 1771 and its distribution only in Eurasia (Suciu 2008).

Reproduction takes place in the Danube and surrounding rivers (Bloesch, Jones et al. 2006). It was always rare in the middle and upper Danube and has recently extirpated from the upper Danube and the upstream (Hungarian-Slovakian) stretch of the middle Danube, due to the construction of the two hydropower dams. The last known specimen from the Slovakian at Mohacs in 1965 (Guti 2006). Nowadays, the majority of the population migrate as far as the Iron Gate II Dam (Fig. 2), with only few individuals succeeding in passing through the shipping locks (Bloesch, Jones et al. 2006).

Fig. 2. The Danube River basin - Black lines numbered 1 and 2 represent the locations of hydropower plants Iron Gate I and II (Wikipedia).

2. Biology of the species

Stellate sturgeon is an anadromous species (Lucas and Baras 2001), spending most of its life feeding and overwintering at sea. Adults generally occur in deeper areas (10-40 m deep) far from shore, while juveniles utilise suitable feeding habitats near the river mouth.. Adults feed mainly on marine benthic fauna and only up to 2% on small fish (Shubina, Popova et al. 1989). Juveniles feed on benthic invertebrates such as crustaceans, molluscs and larval stages of insects, while the nutrition of early larval stages involves mainly plankton (Shubina, Popova et al. 1989).

Stellate sturgeons cease feeding after the onset of the spawning migration. They return to the sea after spawning, where they again begin to feed actively (Reinartz 2002). Stellate sturgeon can reach a maximum length of 218 cm and a maximum weight of 54 kg, but they usually range from 100 to 120 cm and 6 to 8 kg. The differences between the sexes are slight, but females are larger than males of the same age (Shubina, Popova et al. 1989), (Reinartz 2002)**.**

3. Life history and ecology

Spawning migration

A. stellatus migrates between freshwater and salt- or /brackish water in the Black, Azov and Caspian Sea (Billard and Lecointre 2000).

Although sturgeon feed as adults in the Black Sea, they return to the rivers for spawning and may home specifically to the area of the river where they were born. Homing fidelity has still to be proven for sturgeons, but is thought to be significant (Suciu 2008). As with other anadromous species, this behaviour of sturgeons potentially allows significant population structuring, the degree of this structuring depends on the accuracy of return to natal areas. In another sturgeon species, the Atlantic (*Acipenser oxyrinchus)* (Stabile, Waldman et al. 1996) found significant differences in mtDNA haplotype frequencies in different Gulf of Mexico drainages, indicating substantial geographic structuring of populations.

Migration of sturgeons can be observed throughout the year in the lower Danube basin, but two peaks have been observed. Depending on the distance that spawning fish must travel, there are two peaks of migration, one in fall and one in spring (Shubina, Popova et al. 1989), (Suciu 2008). If the spawning sites are located in the downstream sector of the river, the spawners ascend the river in spring, from March to May (Bacalbasa-Dobrovici 1991).

If the sites are far upstream, they first undertake a migration in fall (October, November) (Bănărescu 1964; Manea G. 1966), overwinter in the river, and complete their migration in the following spring (Hensel and Holčík 1997).

Being the smallest of all anadromous sturgeon species living in the Black Sea, the stellate sturgeon starts its spring migration at higher temperatures than Beluga and Danube sturgeon, enabling it to save energy during the long route. Thus, stellate sturgeon spawning migration in the Danube River takes place immediately after the *Huso huso and A. gueldenstaedti* ones (Antipa 1909; Shubina, Popova et al. 1989).

The spring migration starts in March (water temperature 8-11 ºC), with a peak intensity in April, and continuing throughout May (Shubina, Popova et al. 1989). The fall migration begins in August and lasts until late November (water temperature 6.5 ºC) (Suciu, Onara et al. 2011). The fall migrants overwinter in the river and reproduce the following spring (Hensel and Holčík 1997). A likely wintering area was identified at river km (rkm) 75–76 (St George Branch) (Kynard, Suciu et al. 2002). The upstream migration speed of stellate sturgeons according to (Kynard, Suciu et al. 2002) is 7 – 8 km / day (ground speed).

Annual recruitment and spawning success are highly variable depending on the number of adults migrating into the river, fishing mortality, water temperature and flow regime during the spawning "suitability windows" for day length, water temperature and water discharge / level (Shubina, Popova et al. 1989), (Kieffer and Kynard 2004), (Suciu 2008). An important trigger for sturgeon migrations is represented by the periods of high flow. The higher water levels at such times enable fish to pass through shallow river stretches. Any reduction in river discharge at the time of migration reduces the attractiveness of the river, and thus the number of anadromous spawners (Pavlov 1989).

The stellate sturgeons mature earlier than other sturgeon species; males at 5-6 years and females at 7-10 years (Shubina, Popova et al. 1989).Sturgeons do not spawn every year (Reinartz 2002) due to the fact that the egg development process (vitellogenesis) requires more than one year. Thus, only a small part of the whole population takes part in the annual spawning cohort. Spawning takes place at shorter time intervals in males than in females (Shubina, Popova et al. 1989); each 3 or 4 years respectively (Vecsei, Peterson et al. 2007). Spawning occurs from May to June at water temperature (17 – 23 ºC) in the Danube River (Shubina, Popova et al. 1989), (Ivanov 1987), (Ciolac and Patriche 2005).

Recent data (from 2008) indicates that sturgeon spawning in Danube can occur in even lower temperature than described in the literature if the other requirements are fulfilled (Suciu, Onara et al. 2011).

The spawning sites are characterized by a substrate made of scattered stones, pebbles (Shubina, Popova et al. 1989), clay walls / sills (Antipa 1909) and moderate water velocity (Kynard, Suciu et al. 2002).

The locations of spawning sites of this species in the lower Danube under the changed (post-Iron Gates) migratory and hydrological conditions are unknown (Suciu 2008).

Almost nothing is known about stellate mating and spawning habits (Suciu 2008). Lower Danube River is the last known area (Vassilev 2006) which provides spawning and juvenile rearing habitats as indicated by the results of the Natural recruitment of Young of the Year (YOY) assessed by monitoring of downstream migration of YOY by the Sturgeon Research Group (SRG), within the Danube Delta National Institute (DDNI), each summer since 2000. The data revealed that all four sturgeon species found in the lower Danube are spawning between rKm 100 and Iron Gate II (Danube River Km 863) (Suciu, Onara et al. 2011).

Early life stages

An adult stellate sturgeon female may produce 80,000-180,000 eggs (body weight 10-15 kg) (Shubina, Popova et al. 1989). The fecundity increases with age, and the fecundity increases with age.

The eggs are adhesive and are deposited on beds of stones, pebbles and gravel, which offers good protection from predators and ensures a high level of survival (Kynard and Horgan 2002). When a substrate is not available, eggs are laid on other material, such as shifting sand mixed with clay. A decrease in current velocity can lead to an increased mortality of embryos (Kynard and Horgan 2002). In case of reproduction not taking place for various reasons, the eggs are resorbed, but can be mobilized again for reproduction in adjacent years (Artyukhin 1979).

The incubation spans 60 – 130 hours and depends on water temperature (optimal temperature 17-23 °C) (Ivanov 1987). A study on the effect of temperature on incubation of Atlantic species, *Acipenser transmontanus* and *A. fulvescens* eggs revealed that the optimal survival is between 14 – 17 °C and the incipient mortality occurred at 20 °C (Wang, Binkowski et al. 1985).

The new generation does not remain long in the rivers (Shubina, Popova et al. 1989); the downstream migration of larvae is very rapid, 50 to 60 km per day (Suciu, Rosten et al. 2011). During subsequent development, their capability of active movement increases.

To further understand the early life stages behaviour during downstream migration from spawning grounds, laboratory experiments were performed by DDNI in 2005 and 2007 (Suciu, Paraschiv et al. 2007) . That study represented the only investigation in the early life stages (ELS) of stellate sturgeon. The outcome of the experiments revealed that the yolk sack larvae

began passive drifting downstream from the spawning sites from the first day and lasted for the whole period of the experiments (22 days in year 2007).

After the resorbtion of yolk sac, the larvae feed on zooplankton. From the 4th -5th day larvae exhibited active, pelagic swimming upstream, in search for food (both behaviours are shown in Fig. 3).

At approximately 16 – 17 days old, larvae move from pelagic to benthic habitat use and actively swim at the bottom to feed on benthic animals. Immediately after hatching, the newly released embryos are phototropic; this strong behaviour will change during their development into larvae as shown in Fig. 4.

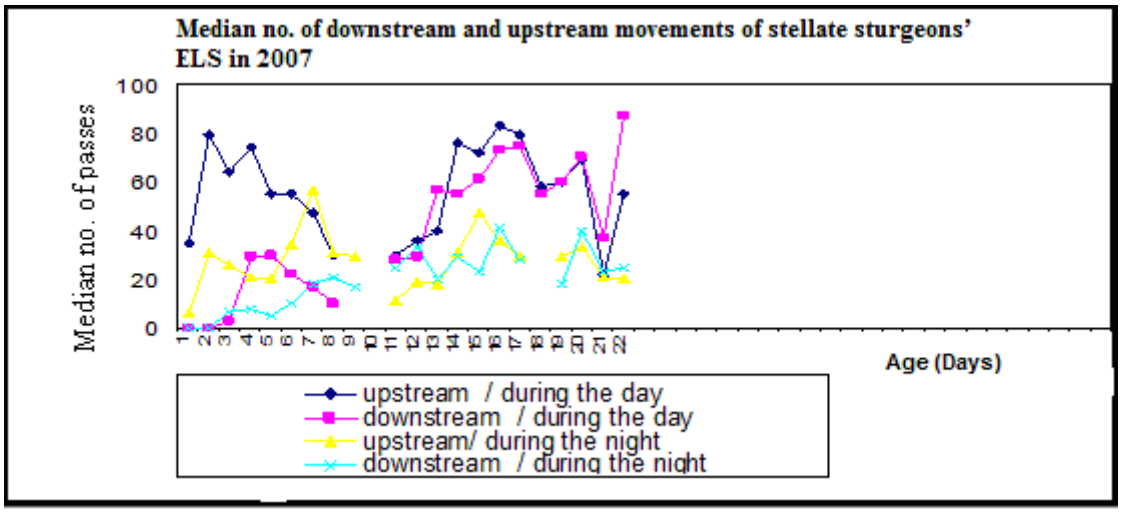


Fig. 3. Median number of downstream and upstream movements of the early life stages of stellate sturgeons, Research conducted in 2007 (N= 15) (Suciu, Paraschiv et al. 2007).

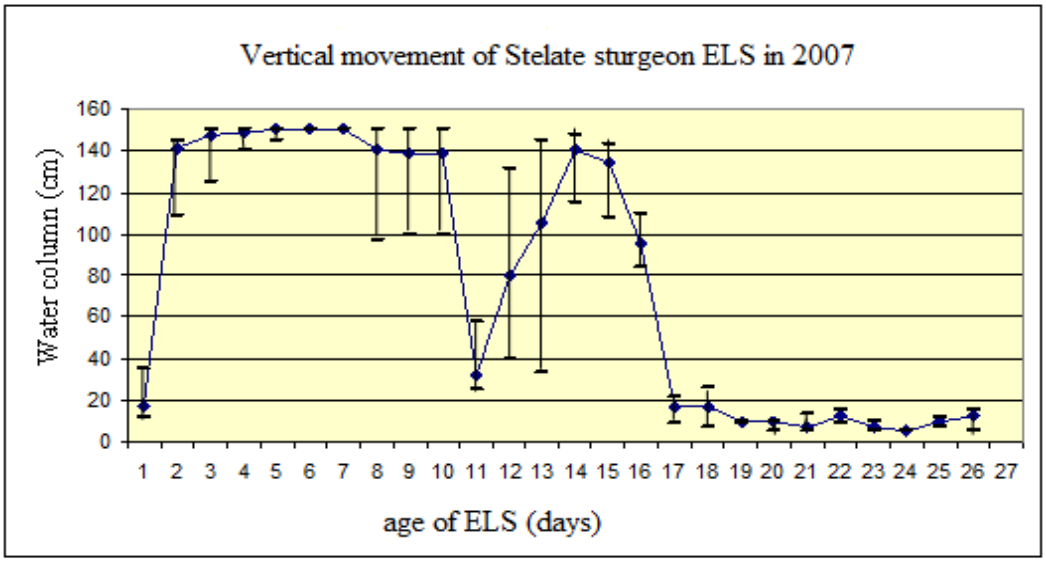


Fig. 4. The vertical movement of stellate sturgeons' early life stages in the experiment of 2007 (N=15) (Suciu, Paraschiv et al. 2007).

4. Hybridisation

The stellate sturgeon easily produces hybrids. In its natural habitats, *A. stellatus* is known to interbreed with *Huso huso, A. gueldenstaedti* (Shubina, Popova et al. 1989). The main explanation of hybridization is that the suitable spawning habitats are lost due to anthropogenic impacts; therefore the different species are confined to fewer suitable sites (Reinartz 2002).

Stock fluctuations

Human impact overview

Sturgeons represent a great economic value for the countries along the Danube. They have been fished mainly for their eggs, the black caviar, which is very expensive in the modern time. The price for 1 kilo of beluga caviar, for instance, rose from 300 Euro in local back markets to approximately 1000 Euro in duty free shops and about 6000 Euro in luxury sales outlets (WWF - Action plan for sturgeons). These aberrant prises for caviar have put pressure on legal or illegal fishing of ripe adults migrating upstream for spawning, allowing the legal and black market to flourish.

Since 1878 (the year of the Berlin Peace agreement), the lower Danube and the Danube Delta have been under the control of the Romanian state.

For a long time, overfishing of sturgeons in this area was extensive. During the communist regime (1948–1989), the centralized economy did not consider ecological criteria in the sturgeon fishery. The situation, however, was not improved in the post-communist period; in fact the number of fishing permits has increased and records on the extent of sturgeon fishing are limited. During the communist period fishing records were extremely extensive in Romania (Bacalbasa - Dobrovici and Patriche 1999).

According to official reports, the decline of sturgeon stock in the lower Danube River is mainly due to overfishing (Fig 5) (WWF- action plan for sturgeons).

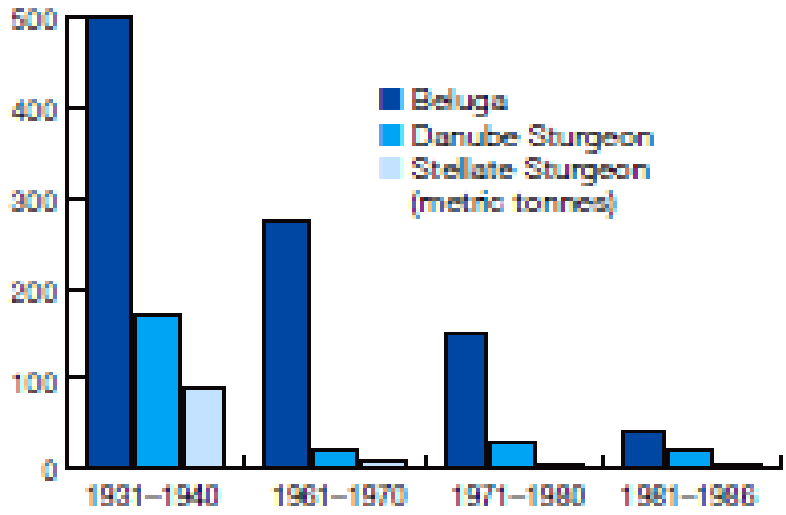


Fig. 5. The decline in sturgeon catches in the lower Danube River by species, due to excessive catches mainly in Romania and Ukraine (Navodaru, Staras et al. 1999) cited by (WWF- Action Plan for sturgeons).

Unfortunately, the official statistic reports for the real stock situation are not always correct because of lack of data about the underground (poacher's) catches. These catches might exceed many times the legal ones (Vassilev 2006). It is believed that previously, at certain periods, poaching represented an increase of up to 90% on the official catch statistics (Bacalbasa - Dobrovici and Patriche 1999), (Navodaru, Staras et al. 1999).

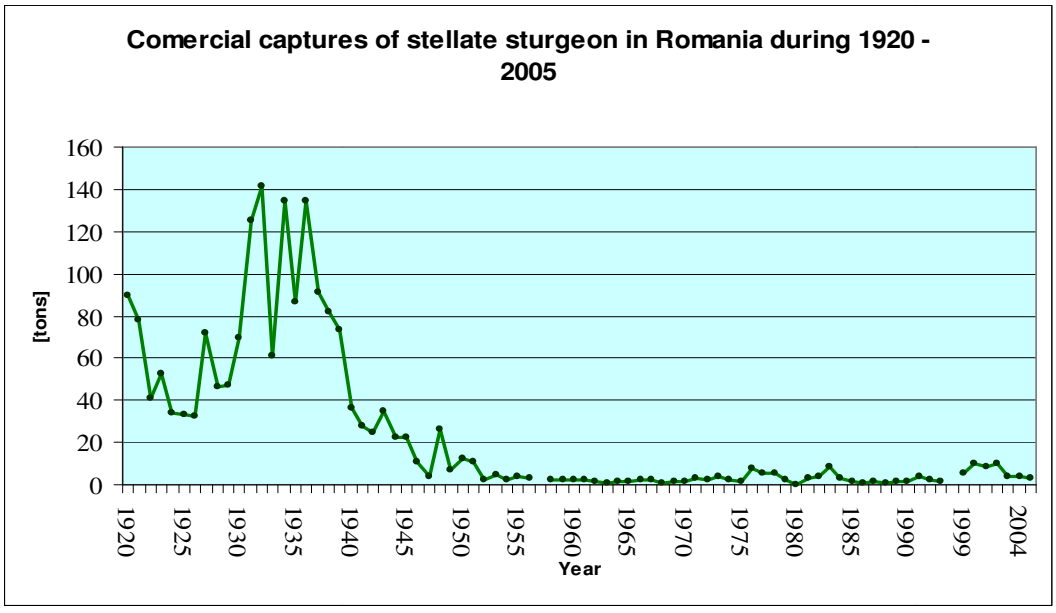


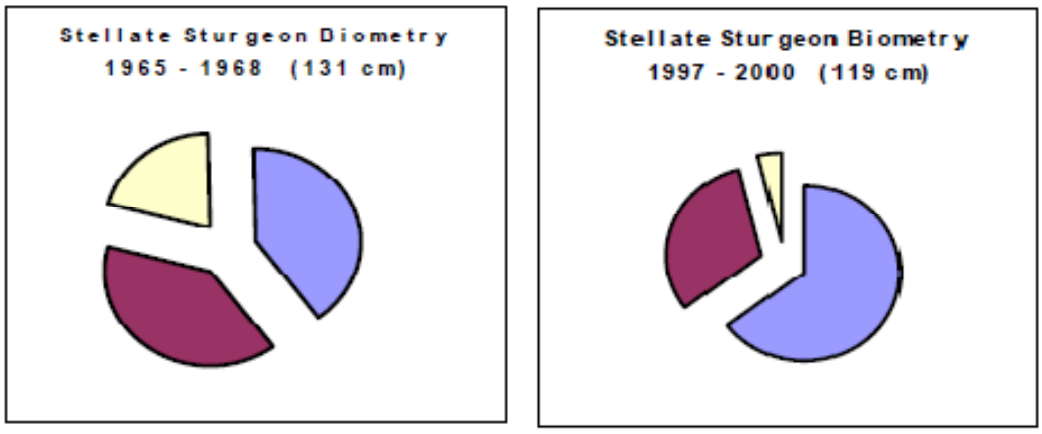
Fig. 6. The commercial catches of stellate sturgeon in Romania during 1920 – 2005 (Manea G. 1966).

The database of commercial catches of stellate sturgeons in Romania during 1920 - 2004 is shown in Fig.6 (Manea G. 1966). Data from 1920 to 1940 represents the total catch in

Romania before the Second World War; this is thought to be reliable. Under the 1947 Treaty of Paris, Romania lost Basarabia and Moldova territories, hence data in the graph before 1947 were registered from the former Romanian territory. After 1947 and until 1989 the communist regime ruled Romania. During that time only the annual planned catches were registered. The unreported captures were hidden very easily (because of the smaller size of stellate sturgeons compared with beluga sturgeons for instance) and brought home as a source of food for families and relatives (Suciu, R.–pers. comm). However, despite this a decrease in the annual catch was still recorded.

A decrease to nil catches of stellate sturgeons was registered by Serbia after completion of Iron Gate Dam II in 1984. The stellate sturgeon catches in (1967–1970) were analysed by Iancovic in 1993. The annual catch was between 1.4–2.0 tons until the first Iron Gate dam was finalised, in 1971, then the catch dropped to just 184 kg. During the following 8 years the species was not recorded in the annual sturgeon catch, with the exceptions of; 1975 when 284 kg was caught, and 1980 when 80 kg was reported. After the completion of Iron Gates Dam II in 1984, the stellate sturgeon disappeared from the middle Danube catch reports (Vassilev 2006).

In addition to stock fluctuations, the age structure of the population has also been affected. Older ripe adults which undertake spawning races upstream Danube were targeted during fishing. This resulted in a long term shift in the population structure; to a smaller size and younger age, losing the large, older spawners. This shift is reflected in Fig 7, which shows that the when mean body length decreased from 131 to 119 cm; data from Ceapa *et. al*. in 2002 cited by (Bloesch 2006).



Legend: light yellow: 14-23 years old; dark-red: 8-18 years old; grey-blue: 3-11 years old

Fig. 7**.** Change of population structure of *A. stellatus* in the Danube River. Data from Ceapa *et al.* (2002) cited by (Bloesch 2006).

A telemetry study to identifying the fishing pressure on stellate sturgeons was undertaken by (Kynard, Suciu et al. 2002). Tag returns of 38 % in 1998 and 27 % in 1999 indicated a severe over-harvest of the species. The authors predict the imminent collapse of stocks in the lower Danube, if the present rate of harvest continues (Bloesch, Jones et al. 2006).

The more recent development evolution of sturgeon stocks in the N-W Black Sea and the lower Danube River has been surveyed by the DDNI., Using fishery independent (JPI – juvenile production index) and fishery dependent (age structure) information, it has been observed that the stellate sturgeon cohort from 2003-2004 consisted mainly of first time spawners (6 – 8 years old). The older age classes are still lacking due to unsustainable legal and illegal fishing during 1990 – 2000 (Fig. 8) (Suciu, Paraschiv et al. 2007).

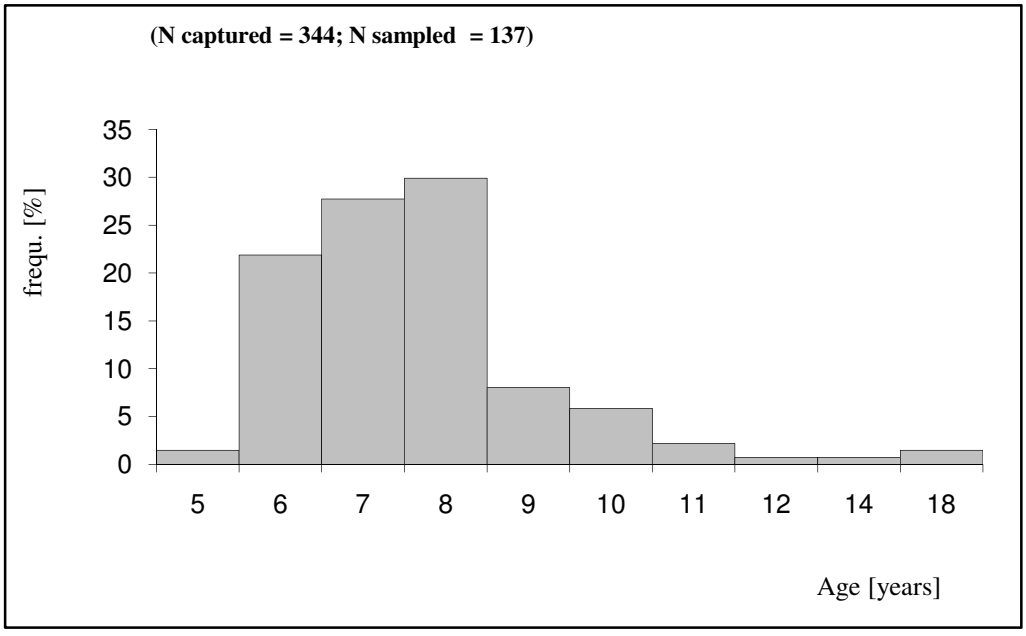


Fig. 8. The age class structure of stellate sturgeon in 2003 – 2004 (Suciu, Paraschiv et al. 2007).

The Juvenile Production Index - Natural recruitment of Young of the Year stellate sturgeon in the Lower Danube River (2000 – 2010), (Fig. 9) monitored by DDNI every summer since 2000 has revealed the lack of natural recruitment in 2006 and 2007.

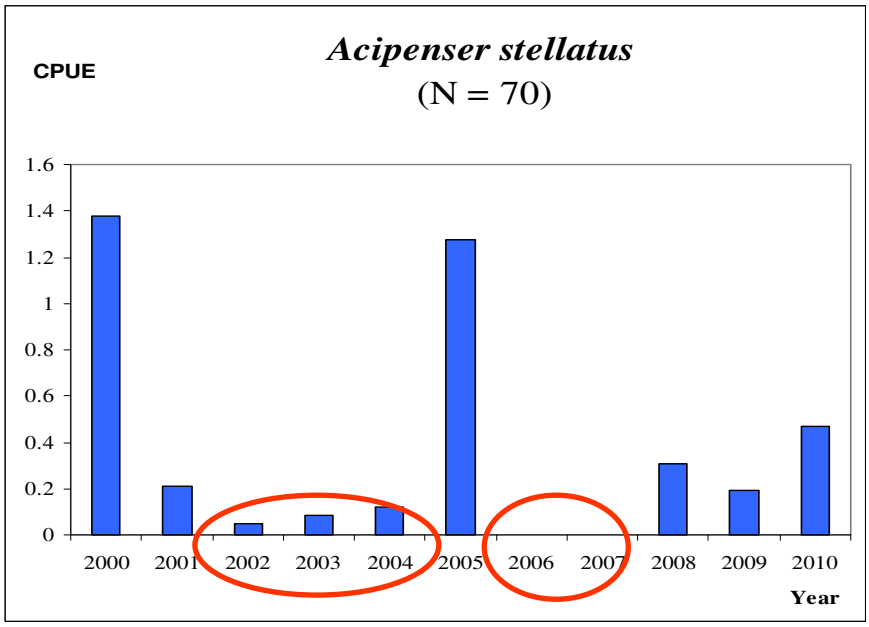


Fig. 9. Juvenile Production Index (JPI) graph: Natural recruitment of A. stellatus in the lower Danube River during 2000 – 2010 - Assessed by monitoring the downstream migration of YOY (Suciu, R. - unpublished data).

Since drastically decreases of stocks were observed, the Black Sea stock of stellate sturgeon have been listed in Annex II of the Convention on International Trade with Endangered Species (CITES) (called also Washington Convention) in April 1998 and as an endangered species in the Red list of IUCN, since 2000 (IUCN Red List).

Supportive stocking programme

Knowledge of the number and geographical distribution of the constituent populations of sturgeons is a prerequisite for management and conservation in order to avoid overexploitation of specific populations. Such information is also a basic requirement for the establishment and use of hatchery-reared juveniles for possible supplementation. The extensive literature on salmonid species has confirmed how natal homing results in a complex population structure that has an important bearing on management activities. Thus in spite of adult admixture in sturgeons, reproductive isolation due to natal homing could result in genetically distinct populations (Ferguson, Suciu et al. 2000).

Depletion of natural stocks of sturgeons has led to increasing use of supplementation by hatchery-produced juveniles to increase production (Ferguson, Suciu et al. 2000).

There is still a significant lack of knowledge regarding species identification, migration behaviour and natural reproduction (Reinartz et al. 2003) cited by (Lenhardt, Jarić et al. 2008).

A ten years sturgeons Moratorium was issued by Romanian Government in 2006. This allows only of a small number of wild adult sturgeons to be captured, with special permits, and used for artificial breeding and development of sturgeon aquaculture. All the brood stock will be PIT tagged and after breeding will be released back into the Danube (Suciu, Paraschiv et al. 2007).

To preserve the genetic diversity, a minimum effective number of 100 brood fish / generation interval should be used for artificial propagation. The annual effective number (N_e) is 14 for stellate sturgeon. Eggs from each female are divided into a number of batches according to the number of males and fertilized separately with sperm from each male (Sturgeons of Romania and CITES).

Table 1. Number of fingerlings of stellate sturgeons stocked into the Danube within the Romanian Supportive Stocking Programme (Suciu, R.-pers. comm).

Year 2005	Year 2006	Year 2007	Year 2008	Year 2009	Total no. of fishes (2005 - 2009)
7881	53300	-	25000	30000	116181

The juveniles obtained by artificial propagation are raised to an average length of 15 cm and are individually tagged with Coded Wire Tags (CWT) before releasing (Paraschiv, Suciu et al. 2006). They are stocked in the Danube close to the location where their parents were captured. All fishing is forbidden in the area prior to stocking (Suciu, Paraschiv et al. 2007). The number of stellate sturgeons juvenile stocked / year in the Lower Danube River within the Romanian Supportive stocking programme is shown in Table 1.

During experimental fishing, while monitoring the natural recruitment of sturgeons (June – Sept. 2007), seven CWT tagged stellate sturgeons were captured in the Danube at rkm 123 and 100. It was found that these fishes were stocked into the Danube in 2006, at rKm 40 on the Borcea branch.

These fishes gave valuable indication on the growth rate of stellate sturgeon:

- Total length of fish released in Dec. 2006 was 17 cm

- Total length of fish recaptured after 6 months = 37.75 cm and after 8 months = 43.18 cm. Gathering the data, the growth rate of stellate sturgeons is 3.27 – 3.45 cm / month (Suciu, Paraschiv et al. 2007). This is the first post stocking evaluation.

An evaluation of the survival rate of stellate sturgeons stocked in the river during (2005 - 2009) and at the Black Sea coast will be needed to improve the actual practice of the Danube Supporting Stocking Programme.

Baseline genetic information

A first attempt to study the phenotype of stellate sturgeon larvae and juveniles from the Volga River were undertaken by (Prokes, Barus et al. 1996). They observed and described five different groups of fish (see Fig. 10), according to the shape and size of black spots on the ventral side of their heads.

However, genetic studies were not undertaken on the analysed specimens to survey whether the phenotypic differentiations have a genetic basis.

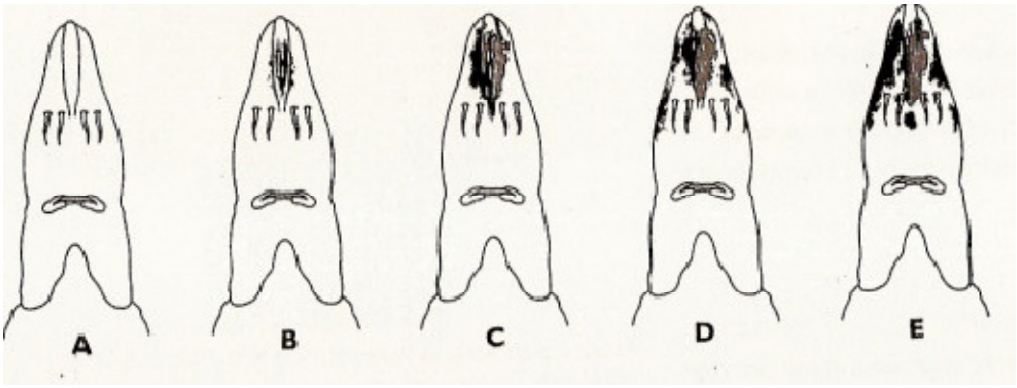


Fig. 10 Variation in black pigmentation of the ventral head section of *A. stellatus,* larvae and juveniles (Prokes, Barus et al. 1996).

According to (Doukakis, Erickson et al. 2008), studies combining tagging and genetic analyses are mandatory for planning an appropriate management of an endangered species. Molecular analysis can be an effective tool in elucidating stock structure and in inferring gene flow of fishes (Stabile, Waldman et al. 1996). Considering this, in 2000 within the frame of the "Royal Society Joint Projects with Central / Eastern Europe and the former Soviet Union", one project on "Genetic population structure of endangered sturgeon species of Lower Danube" was carried out. The two partners of the project (Danube Delta National Institute – Sturgeon Research Group, from Romania and Queen's University of Belfast - Medical Biology Centre from Great Britain), undertook studies on the genetic diversity of stellate

sturgeon in the lower Danube River. One of the aims of this joint study was to survey the extent and distribution of genetic variation of stellate sturgeon and to incorporate the genetic information obtained into a management strategy for this species.

Unfortunately, due to unoptimal sample quality sampling, no significant relationship was found between the distributions of groups of haplotypes with the position in the river of the fish where the samples were obtained from.

However, microsatellites and mtDNA analyses (*ND5/6* region) brought to light the diversity of stellate sturgeons in the lower Danube. Substantial polymorphism was observed at the microsatellites loci with up to 19 alleles, while 6 haplotypes on *ND 5/6* region of mtDNA were found.

In 2007, molecular genetic analyses were performed (Onara, Paraschiv et al. 2007), on the brood stock used for artificial propagation within the Supportive Stocking Programme. This time the sampling procedure was improved; all adults were PIT tagged, and the biometry parameters as well as the place of capture were registered in a database. The mtDNA analyses revealed that stellate brood fish having rare *cyt b* and *ND 5/6* region haplotype migrate early, their spawning grounds are more distant from the sea and were therefore, historically more vulnerable.

Assessing the genetic diversity of the population of *A. stellatus* at seven microsatellites loci, (Dudu, Georgescu et al. 2008) found a high level of polymorphism; 7 - 13 alleles were observed.

Molecular genetics is also a valuable tool in differentiation of early life stages of fish that share the same habitat at the same time, where visual identification is difficult (Ludwig 2008). Species specific markers from PCR-RFLP analyses based on mitochondrial DNA are found to be effective for species identification (Ludwig 2008). All fingerlings from 2000-2010 were genetically confirmed as *A. stellatus* prior to further investigations. Juveniles of stellate sturgeons caught as part of the Monitoring of Natural recruitment of *A. stellatus* in the lower Danube River programme (2000 - 2010), firstly identified by morphological features, were investigated by PCR-RFLP analyses of mtDNA *cyt b* region according to (Ludwig 2008).

Combined tagging and genetic studies can yield results that challenge long-held assumptions about sturgeon biology and reveal new management needs. For example, green sturgeon (*A. medirostris*) was traditionally thought to be primarily marine, moving into freshwater for short periods to spawn. However, telemetry work revealed that the species can spend up to six consecutive months in fresh water (Erickson, North et al. 2002).

Materials and methods

Sample collection

Tissue for DNA preparation was obtained by the Sturgeon Research Group (**SRG**), within the Danube Delta National Institute and / or through collaboration with professional fishermen. The samples were collected from different groups of stellate sturgeons as shown in Table 2.

Table 2. *Acipenser stellatus* DNA samples / tissues used in the current study.

Year	Age	Season of capture	No. of individuals
1998-99	Adults	Autumn (September- October)	15
2007	Adults	Spring (1st of April-10th of May)	22
2010	Adults	Spring (24th-29th of April)	12
	YOY (young of the year)	Summer (22nd of June-7th of July)	16
Total			65

Adult stellate sturgeons were captured during fall and spring migration races by professional fishermen using special bottom drifting trammel nets while young individuals were caught by Sturgeons Research Group (SRG) within Danube Delta National Institute, using trammel net, 90 x 1.8 m, 20 mm mesh size, special mount for bottom drifting. Adult stellate sturgeons were captured both in the Black Sea and in the Danube River while the Young of the Year (YOY) were captured and sampled in the Danube River at Km 123 (see Fig.11), the nursery site identified and monitored by SRG since year 2000. Location of capture / release places in the river is given as distance in [rKm] from the Black Sea on the Sulina branch (Fig. 11).

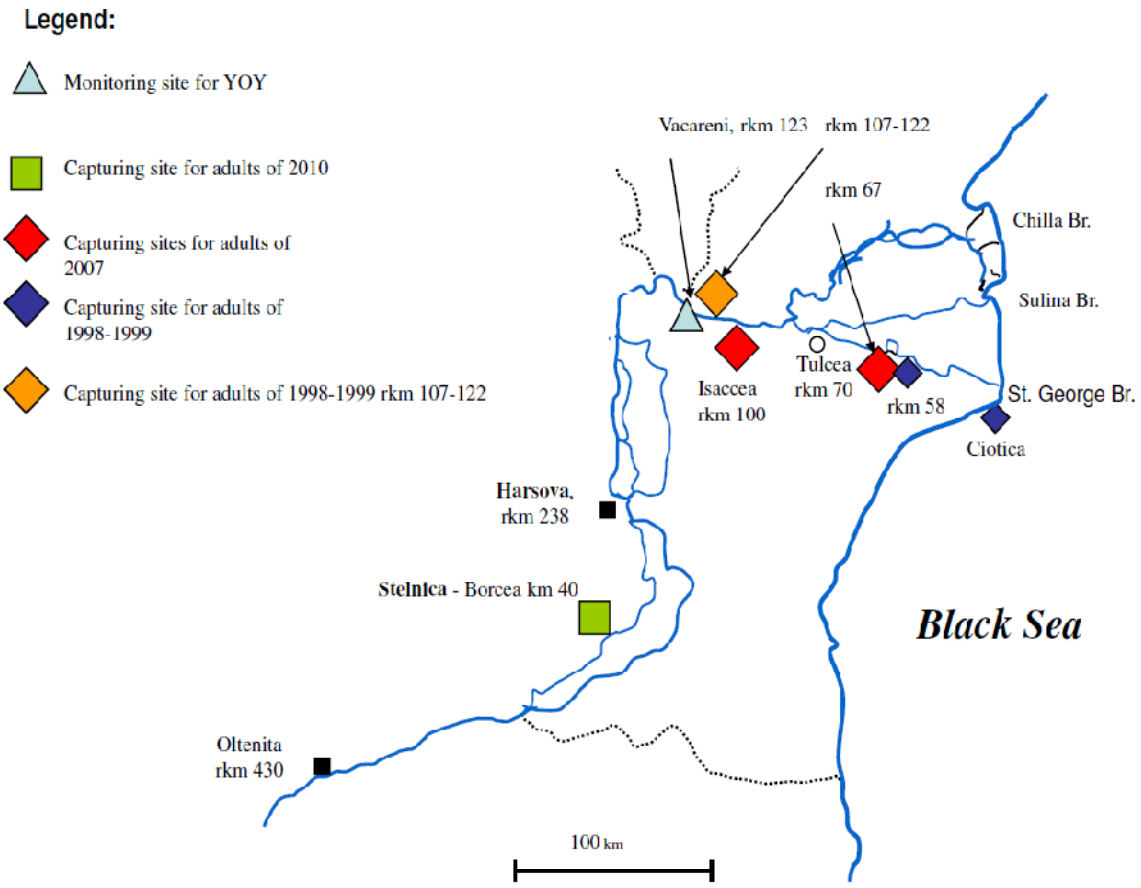


Fig. 11. Map of the Lower Danube River sector and the NW part of the Black Sea showing approximate sampling location of *Acipenser stellatus* individuals.

The adults of 1998-1999 chosen for the study were included in the batch of samples because they were the only confirmed representatives of the fall migration race (captured in September-October).

The adults from 2007 represent the broodstock used for breeding for both aquaculture and supportive stocking programme and were released back in the river afterwards. The adults caught for propagation purposes in 2010 were artificially propagated and released in the river afterwards but because of lack of financing, thus there was no stocking programme in 2010.

The wild YOY individuals were born in the river in 2010. The year 2010 was very successful year for sturgeon spawning in the Danube River.

In this respect we collected and 16 individuals of stellate sturgeon were collected between 22nd of June and 7th of July. The samples details (code, the place and date of capture) and the biometry data are shown in Table 3.

Table 3**.** Data base of analysed samples showing the place and date of capture of specimens used for this study.

Sample	Capturing place [RKm]	Age	Capturing date	Total Weight (g)	Total Length (cm)	Standard Length (cm)
R100	113	Migrating adult	10-Sep-99	9000	-	-
R101	115	Migrating adult	10-Sep-99	5000	-	-
R102	113	Migrating adult	10-Sep-99	5500	-	-
R103	113	Migrating adult	12-Sep-99	5000	-	-
R104	113	Migrating adult	12-Sep-99	7000	-	-
R107	113	Migrating adult	13-Sep-99	7000	-	-
R108	115	Migrating adult	13-Sep-99	7500	-	-
R115	122	Migrating adult	23-Sep-99	-	117.0	-
R116	122	Migrating adult	23-Sep-99	-	118.0	-
R120	107	Migrating adult	30-Sep-99	-	44.0	-
R124	Ciotica (Black Sea coast about 10 km South from the mouth of the St. George branch)	Adult	30-Sep-99	180	42.0	-
R125	Ciotica (Black Sea coast about 10 km South from the mouth of the St. George branch)	Adult	30-Sep-99	160	40.0	-
R126	58	Migrating adult	28-Sep-98	7000	119.0	-
R127	58	Migrating adult	30-Sep-98	8750	130.0	-
R128	58	Migrating adult	1-Oct-98	6250	118.0	-
7_4_5	Isaccea (Rkm 100)	Migrating adult	1-Apr-07	8000	-	-
7_4_6	Isaccea (Rkm 100)	Migrating adult	7-Apr-07	10000	-	-
7_4_9	Isaccea (Rkm 100)	Migrating adult	16-Apr-07	-	-	-
7_4_15	Isaccea (Rkm 100)	Migrating adult	4-Apr-07	4500	-	-
7_4_16	Isaccea (Rkm 100)	Migrating adult	5-Apr-07	5500	-	-
7_4_17	Isaccea (Rkm 100)	Migrating adult	3-Apr-07	6000	-	-
7_4_18	Isaccea (Rkm 100)	Migrating adult	4-Apr-07	5000	-	-
7_4_19	Isaccea (Rkm 100)	Migrating adult	5-Apr-07	4000	-	-
7_6_10	Isaccea (Rkm 100)	Migrating adult	3-May-07	-	108.0	90.0
7_6_12	Isaccea (Rkm 100)	Migrating adult	16-May-07	5000	108.0	95.0
7_5_2	km 67 on St. George branch	Migrating adult	10-May-07	-	-	-
7_5_3	km 67 on St. George branch	Migrating adult	10-May-07	-	-	-
7_5_4	km 67 on St. George branch	Migrating adult	10-May-07	-	-	-
7_5_6	km 67 on St. George branch	Migrating adult	10-May-07	-	-	-
7_5_7	km 67 on St. George branch	Migrating adult	10-May-07	-	-	-
7_5_8	km 67 on St. George branch	Migrating adult	10-May-07	-	-	-

7_5_9	km 67 on St. George branch	Migrating adult	10-May-07	-	-	-
7_5_10	km 67 on St. George branch	Migrating adult	10-May-07	-	-	-
7_5_11	km 67 on St. George branch	Migrating adult	10-May-07	-	-	-
7_5_12	km 67 on St. George branch	Migrating adult	10-May-07	-	-	-
7_5_13	km 67 on St. George branch	Migrating adult	10-May-07	-	-	-
7_5_14	km 67 on St. George branch	Migrating adult	10-May-07	-	-	-
10_6_11	Borcea branch Rkm 37-47	Migrating adult	24-Apr-10	6000	115	98
10_6_15	Borcea branch Rkm 37-47	Migrating adult	26-Apr-10	11000	141	120
10_6_20	Borcea branch Rkm 37-47	Migrating adult	29-Apr-10	6000	112	95
10_9_7	Borcea branch Rkm 37-47	Migrating adult	29-Apr-10	6000	-	-
10_9_1	Borcea branch Rkm 37-47	Migrating adult	29-Apr-10	12000	135	110
10_9_2	Borcea branch Rkm 37-47	Migrating adult	29-Apr-10	11000	147	118
10_9_3	Borcea branch Rkm 37-47	Migrating adult	22-Apr-10	4500	110	95
10_9_4	Borcea branch Rkm 37-47	Migrating adult	22-Apr-10	5000	112	97
10_9_5	Borcea branch Rkm 37-47	Migrating adult	22-Apr-10	7000	125	105
10_9_6	Borcea branch Rkm 37-47	Migrating adult	22-Apr-10	5000	110	98
10_9_8	Borcea branch Rkm 37-47	Migrating adult	25-Apr-10	10000	127	106
10_9_9	Borcea branch Rkm 37-47	Migrating adult	26-Apr-10	5500	109	95
10_20_8	Vacareni (Rkm 123)	YOY	22-Jun-10	6	12.3	9.5
10_20_9	Vacareni (Rkm 123)	YOY	22-Jun-10	5	10.6	7.8
10_21_7	Vacareni (Rkm 123)	YOY	25-Jun-10	11	15.1	11.0
10_21_13	Vacareni (Rkm 123)	YOY	25-Jun-10	9	15.3	10.9
10_22_2	Vacareni (Rkm 123)	YOY	26-Jun-10	6	12.6	9.3
10_22_19	Vacareni (Rkm 123)	YOY	29-Jun-10	1	6.7	5.0
10_22_20	Vacareni (Rkm 123)	YOY	29-Jun-10	5	12.2	9.1
10_23_7	Vacareni (Rkm 123)	YOY	30-Jun-10	1	7.3	5.5
10_23_14	Vacareni (Rkm 123)	YOY	1-Jul-10	2	7.5	5.6
10_23_22	Vacareni (Rkm 123)	YOY	2-Jul-10	1	6.1	4.7
10_24_2	Vacareni (Rkm 123)	YOY	6-Jul-10	3	9.1	6.7
10_24_3	Vacareni (Rkm 123)	YOY	6-Jul-10	6	11.5	8.6
10_24_4	Vacareni (Rkm 123)	YOY	7-Jul-10	2	8.5	6.3
10_24_5	Vacareni (Rkm 123)	YOY	7-Jul-10	4	10.3	7.8
10_24_6	Vacareni (Rkm 123)	YOY	7-Jul-10	3	9.3	6.3
10_24_7	Vacareni (Rkm 123)	YOY	7-Jul-10	3	10.5	7.7

The genomic DNA extracted from anal fin collected in years 1998-99 and 2007 was stored at -20 ºC until analysis.

Primers and analytical software used

1. Mitochondrial DNA

Primers for *cyt b* region, previously used in identification of 22 acipenseriform species (Ludwig, Debus et al. 2002), used in this study are mentioned in table 4.

The corresponding sequences of control region (*D-loop*) of *A. stellatus* mtDNA, available in GenBank database were aligned and analyzed using BioEdit software. Primers' characteristics and compatibility were estimated using software OligoExplorer1.2 and OligoAnalyzer1.2 (genelink) (see table 4).

Tabel 4. Oligonucleotide primers used for amplifying the target sequence.

Primer	**Sequence (5'-3')**	**Orientation**	**GenBank Acc.No.**	**Amplicon expected size (bp)**
Cyt b F	CGTTGTHWTTCAACTAYARRAAC	sense (forward)	AJ 585050	1203
Cyt b R	CTTCGGTTTACAAGACCG	antisense (reverse)	AJ 585050	
CrFs	GTAGTAAGAGCCGAACATCC	sense (forward)	AF 168513 - AF 168545	450
CrRs	GTCCTGCTTTTGGGGTTTGA	antisense (reverse)	AF 168513 - AF 168545	

PCR

All PCR amplifications were performed in a GeneAmp PCR System 9700 termocycler (Applied Biosystems).

cyt b

50 ml reaction volume containing 1U of GoTaq polymerase (Promega M3005), 10 µl 5x buffer, 200 µM dNTP, 100 µM of each primer and 200 ng DNA. Reaction volume was filled with sterile deionised water.

D-loop region

50 µl reaction volume containing 1U of GoTaq polymerase flexi (Promega M8305), 10 µl 5x buffer flexi, $MgCl_2$ 2mM, 200 µM dNTP, 100 µM of each primer and 200 ng DNA. Reaction volume was filled with sterile deionised water.

Common protocol

A denaturing step for 5 min at 94 °C, followed by 35 cycles of 94 °C for 1 min, 50 °C for 1 min, 72°C for 1 min and a final extension at 72 °C for 2 min.

PCR products were electrophoresed in 0.8% agarose (Sigma-Aldrich A9539) gel in 0.5 x TBE buffer and visualized with ethidium bromide staining.

RFLP

Several restriction enzymes were used in this study (table 3) to distinguish the polymorphisms. Maps of restriction sites of target sequences of *A. stellatus* mt-DNA, estimated by comparing of available corresponding sequences in GenBank (see fig. 12 a, b) were generated using NEBcutter V2.0 **(**NEBcutter**).**

Table 5. Enzymes selected for polymorphism analysis.

Target mtDNA region	**Restriction Enzyme tested**	**Recognition site**	**Polymorphism**
Cyt b	Tru9I (Promega R7011)	T▼TA A A AT▲T	no
	AseI (NEB R0526)	A T ▼ T A A T T A A T ▲ T A	no
	BsrSI (Promega R7241)	ACTG GN▼ TGAC▲CN	no
	HphI (NEB R0158)	GGTGANNNNNNNN ▼ CCACTNNNNNNN ▲ N	yes
	RsaI (Promega R6371)	GT▼AC CA▲TG	yes
	HinfI (Promega R6201)	G▼ANTC CTNA▲G	no
	AluI (Promega R6281)	AG▼CT TC▲GA	no
	DdeI (Promega R6291)	C▼TNAG GANT▲C	no
	HaeIII (Promega R6171)	GG▼CC CC▲GG	no

D-loop	AseI (NEB R0526)	A T ▼ T A A T T A A T ▲ T A	no
	RsaI (Promega R6371)	GT▼AC CA▲TG	no
	HaeIII (Promega R6171)	GG▼CC CC▲GG	yes
	AluI (Promega R6281)	AG▼CT TC▲GA	no
	Tru9I (Promega R7011); Mse (NEB RO525)	T▼TA A A AT▲T	no
	BsrSI (Promega R7241)	ACTG GN▼ TGAC▲CN	yes
	BslI (NEB R0555)	C C N N N N N ▼ N N G G G G N N ▲ N N N N N C C	yes
	BamHI (Promega R6021)	G▼GATCC CCTAG▲G	no

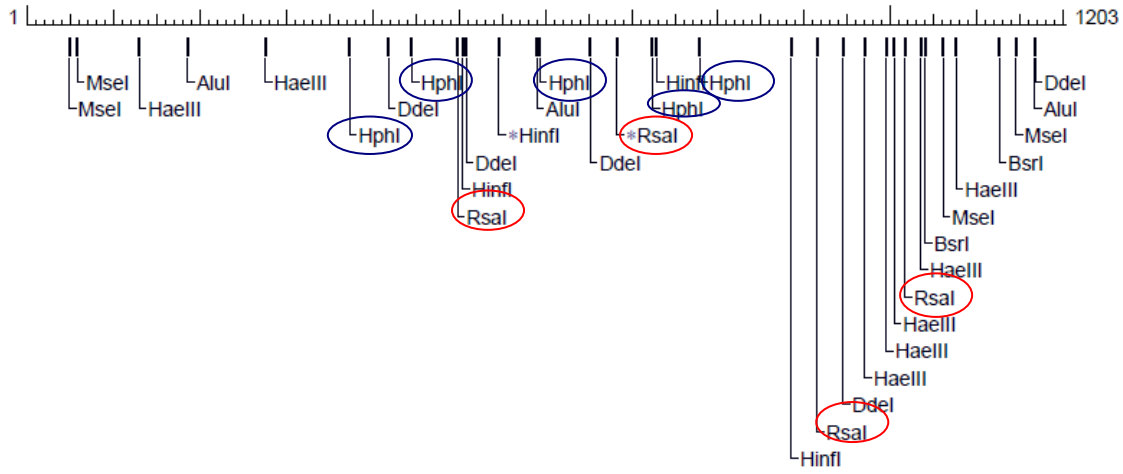


Fig 12 a. Restriction enzyme map amplicon *cyt b*, variable sites identified in GenBank sequences AJ 585050, AY846680 – 84670, AJ249693, GU647226. FJ974042, AF006131, AF006170, AF006142.

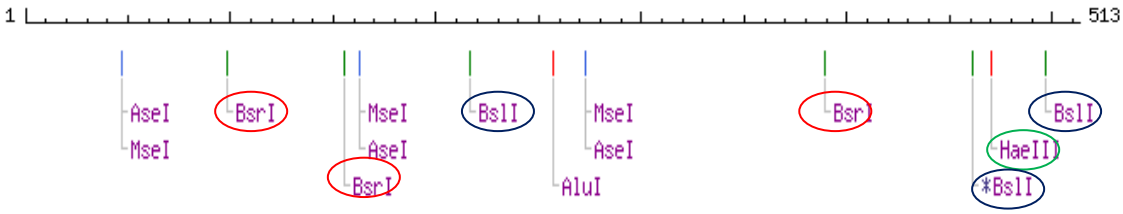


Fig 12b. Restriction enzyme map amplicon *D-loop*, variable sites identified in GenBank sequences AF168513 - AF168545.

Enzymatic digestion was performed overnight at temperatures recommended by the manufacturer in a final volume of 10 ml containing 1U of restriction enzyme. Samples subjected to digestion were separated by horizontal electrophoresis in 2.5% agarose gel in 0.5 x Tris buffer / boric acid / EDTA. DNA was visualized under UV light (302 nm) after reaction with ethidium bromide.

2. Microsatellites DNA

Five microsatellite loci: LS-19, LS-34, LS-54, LS-57, LS-68, initially developed for *Acipenser fulvescens (*May, Krueger et al. 1997) and another two loci Aox-23, Aox-45 previously developed for Atlantic sturgeons (*Acipencer oxyrinchus oxyrinchus)* were obtained from DNA sequences available in the GenBank Database (accession nos. AF067811 and AF067813).

The PCR conditions were optimized according to (Dudu, Georgescu et al. 2008).

An efficient system of fluorescent labelling of forward primers with four distinct dyes PET, VIC, 6-FAM, NED (see Table 6), were employed for microsatellite loci detection.

Table 6. Primer sequences.

Primer	Sequence (5'-3')	Orientation
LS-19	5' 6-FAM-CATCTTAGCCGTCTGGGTAC	sense (forward)
	CAGGTCCCTAATACAATGGC	antisense (reverse)
LS-34	5' VIC-TACATACCTTCTGCAACG	sense (forward)
	GATCCCTTCTGTTATCAAC	antisense (reverse)
LS-54	5'NED-CATCTAGTCTTTGTTGATTACAG	sense (forward)
	CAAAGGACTTTGAAACTAGG	antisense (reverse)
LS-57	5' PET-GCTTGGTTGCTAGTTTGC	sense (forward)
	GTACAGTATGAGACCACAGGC	antisense (reverse)
LS-68	5' NED-TTATTGCATGGTGTAGCTAAAC	sense (forward)
	AGCCCAACACAGACAATATC	antisense (reverse)
Aox-23	5' 6-FAM-CAGTGTGCTAGCTTCTCAATA	sense (forward)
	GTTAGCTTAACCATGAATTGTG	antisense (reverse)
Aox-45	5' PET-TTGTTCAATAGTTTCCAACGC	sense (forward)
	TGTGCTCCTGCTTTTACTGTC	antisense (reverse)

PCR

Amplification of the microsatellite loci was done by two multiplex PCR reactions as follows: 5-Plex reaction for LS-19, LS-34, LS-54, LS-68 and Aox-45 and 2-Plex reaction for Aox-23 and LS-57.

PCR were performed in 25 µL final volume containing: 1X PCR Buffer, Mg Cl_2, 0.4 µL of each primer, 200 µM of each nucleotide, 2 units of AmpliTaq Gold DNA Polymerase (Applied Biosystems 4311806), 20 ng / µL DNA template and sterile deionised water.

Protocol

A denaturating step at 95 ºC for 10 minutes, followed by 45 cycles of 52 ºC, for 30 seconds, annealing at 72 ºC for 30 seconds, extension for 60 seconds; and a final extension for 60 minutes, both at 72 ºC.

The PCR products were mixed with the GeneScan-500 LIZ Size Standard and load into ABI Prism 310 DNA Genetic Analyzer. The fragments were observed with the GeneScan 3.1.2. and Genotyper 2.5.2. Softwares (Applied Biosystems).

The LS-57 and LS-68 microsatellite results were not found suitable for further use. Thus the marker panel for analysis by population genetic software included the four microsatellites LS-19, LS-34, LS-54 and Aox-23 and the mtDNA marker.

3. Statistical analyses

The genotypic composition at four microsatellite loci and mtDNA haplotypes in the six subsamples were written to a DOS text file which served as input to Genepop (Raymond and Rousett 1995). Many of the basic genetic analysis could be performed with "Genepop on the web" and Genepop version 4.1 downloaded from the web (Genepop 4.1).

Cluster analysis and dendrogram construction were performed with the Dispan$^{(C)}$ program suite (Dispan).

For analyses beyond the capabilities of the above mentioned software, the "Convert" program by (Glaubitz 2004) was used to create appropriate input files (Convert website).

Haplotype analyses were performed mainly with the Excel add-in programme" Haplotype Analysis" by (Eliades and Eliades 2009) and Fstat by Jerome Goudet (Fstat).

Results

1. Mitochondrial DNA

Nine restriction enzymes were tested for polymorphism at *cyt b* region (*Tru9I, AseI, BsrSI, HphI, RsaI, HinfI, AluI, DdeI*) and two of them showed intraspecific variation in *A. stellatus*: *RsaI (*4 haplotypes*)* and *HphI* (2 haplotypes) (Fig 13). Eight restriction enzymes were tested for polymorphism at D-loop control region (*Tru9I, AseI, BsrSI, RsaI, AluI, Hae III, BslI, BamHI*) and only three showed polymorphism: *HaeIII* (3 haplotypes), *BslI* (3 haplotypes) and *BsrSI* (3 haplotypes) (Fig 14). Each restriction haplotype was designated by a capital letter and for the composite haplotype by a Roman numeral. The composite haplotypes were assigned by letters indicating the restriction fragment patterns obtained with *RsaI* and *HphI (*for *cyt b)* and *HaeIII, BslI and BsrSI (for D-loop* region).

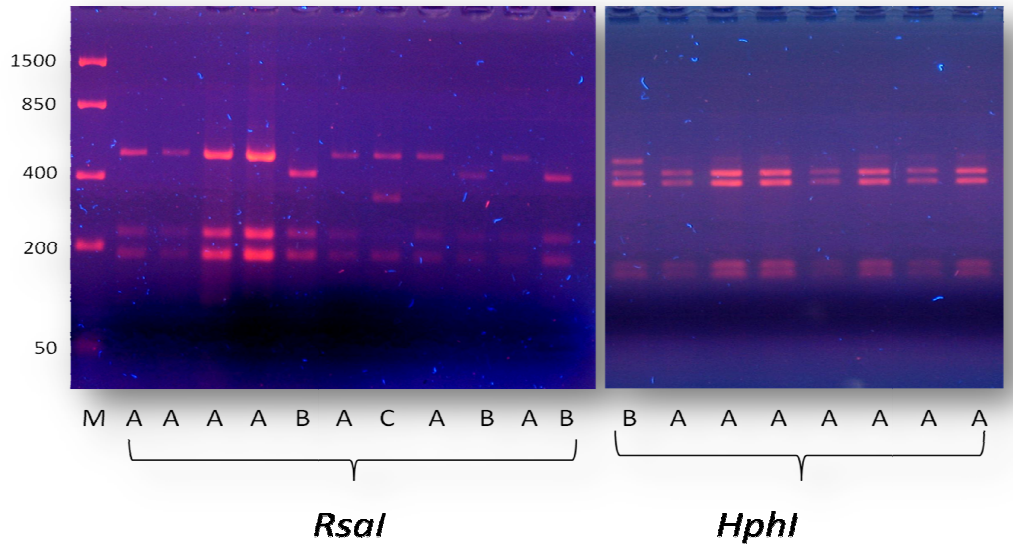


Fig. 13. *Cyt b* fragment digested with *RsaI* and *HphI* (The haplotype designation is mentioned below the picture); M - FastRuler Low Range DNA Ladder, ready-to-use (Fermentas -SM1103).

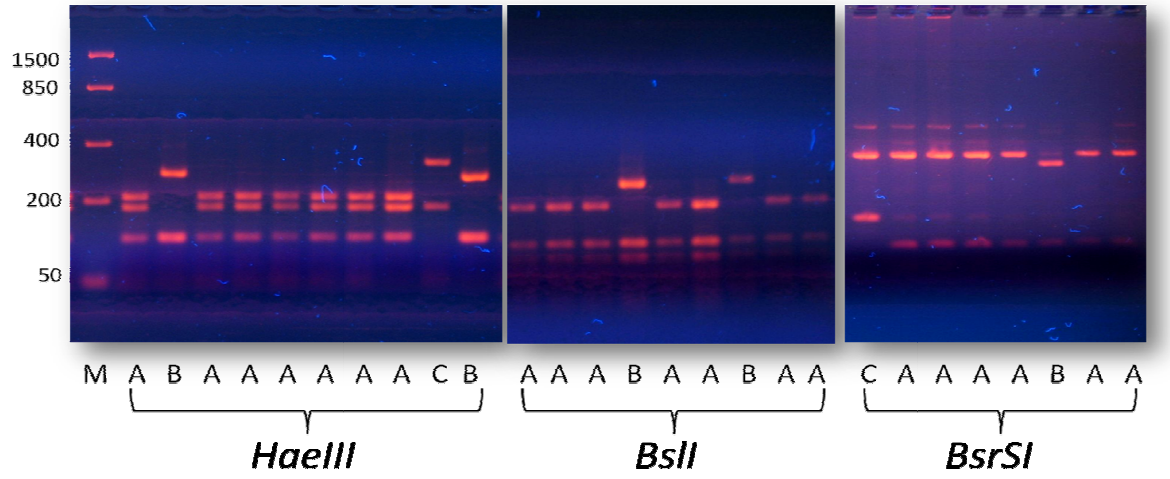


Fig. 14. *D-loop* fragment digested with *HaeIII, BslI* and *BsrSI* (The haplotype designation is mentioned below the picture); M - FastRuler Low Range DNA Ladder, ready-to-use (Fermentas - SM1103).

Twelve composite haplotypes (I - XII) were found in the stellate sturgeon samples under study, and their frequencies are indicated in Table 7.

Table 7. Designation and frequency of stellate sturgeon (N=59) mt DNA composite haplotypes. Letters in haplotype descriptions denote the restriction fragment patterns obtained with *RsaI and HphI (cyt b) and HaeIII, BslI and BsrSI (D-loop region).*

Composite haplotype designation	**Composite mtDNA haplotype (*Cyt b-D-loop*)**	**Haplotype frequency [%]**
I	AAAAA	57.63
II	AAAAB	3.39
III	AAABA	5.08
IV	AABAA	1.69
V	AACAA	5.08
VI	BAAAA	3.39
VII	BAABA	3.39
VIII	BAACA	3.39
IX	BACAA	3.39
X	BBAAA	1.69
XI	CAAAA	10.17
XII	DBAAA	1.69
TOTAL		100.00

2. Microsatellites

Seven microsatellites markers were tested in *Acipenser stellatus*. These involved five tri- (LS-19, LS-34, LS-57) and tetra- (LS-54, LS-68) repeat motif microsatellite markers that were originally developed for Lake sturgeon (*Acipenser fulvescens) (*May, Krueger et al. 1997) and two microsatellites loci, Aox-23 and Aox-45 (tri- and tetra-nucleotide repeat motifs) which were initially developed for the Atlantic sturgeon (*Acipencer oxyrinchus oxyrinchus*) (GenBank Database (accession no. AF067811 and AF067813). The primers were slightly adjusted in order to increase the amplification efficiency for Danube sturgeon species (Ferguson, Suciu et al. 2000), (Dudu, Georgescu et al. 2008).

A high level of polymorphism was observed for the studied population of *Acipenser stellatus*. Two to sixteen alleles where observed with a mean of 10.85 alleles per locus. The most polymorphic locus was Aox-23 while LS-34 showed the lowest polymorphism in the samples included in the current study (see table 9).

Table 9. Number of alleles and ploidy patterns of seven *Acipenser stellatus* microsatellite loci.

Locus	Size (bp)	Alleles number	Ploidy level
LS-19	115 – 145	9	Diploid
LS-34	144 - 147	2	Diploid
LS-54	162 – 192	9	Diploid
Aox-23	92 - 146	16	Diploid
LS-57	171 - 218	14	Polyploid
LS-68	104 - 150	13	Polyploid
Aox-45	94 - 153	13	Polyploid

The microsatellites LS-19, LS-34, LS-54, Aox-23 were diploids and were taken into consideration for further statistic analyses while LS-57, LS-68, Aox-45 were found to be polyploids for stellate sturgeons population in this study and no further analyses were undertaken at these loci. The existence of polyploid loci are likely to reflect a complex genome organisation of sturgeons group which during the group evolution have undergone several polyploidy events (Wirgin, Stabile et al. 1997).

The microsatellite locus LS-19 presented 9 allele with size ranging between 115-145bp. Most of the individuals tested at this locus were heterozygotes. Locus LS-54 showed 9 alleles with size ranging between 162-192bp. Almost 50% of the positive samples were homozygote at this locus.

Two alleles (144 and 147bp) were found for LS-34 and all individuals tested were homozygote at this locus. Only 43% of the DNA samples amplified for this locus. The most polymorphic locus, Aox-23 presented 16 alleles, ranging 92 – 146bp. 31.88% of the positive samples were heterozygote at this locus.

Examples of typical computer generating profiles are shown in Fig. 15 (a-f)

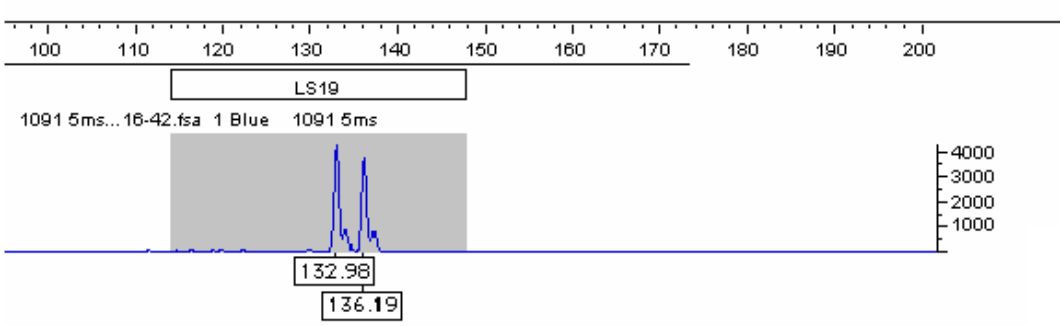


Fig. 15 a. Computer generating profile of PCR amplification products for LS-19.

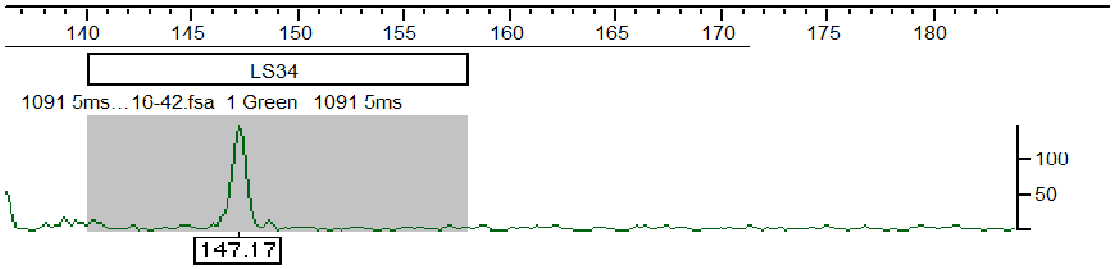


Fig. 15 b. Computer generating profile of PCR amplification products for LS-34.

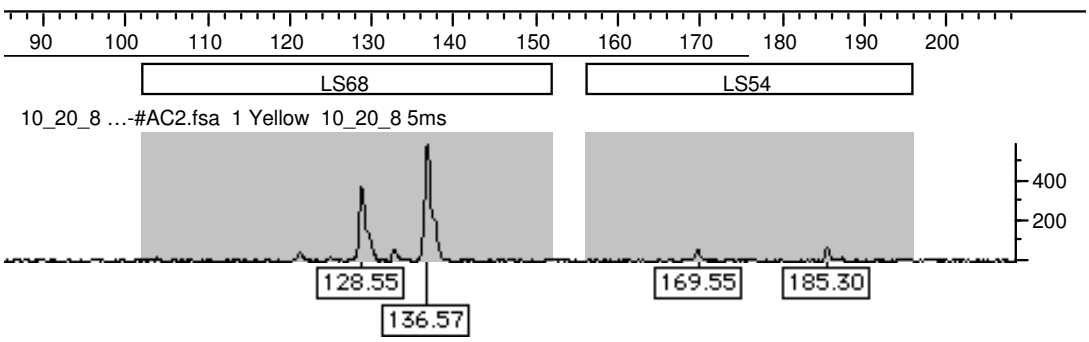


Fig. 15 c. Computer generating profile of PCR amplification products for LS-68 and LS-54

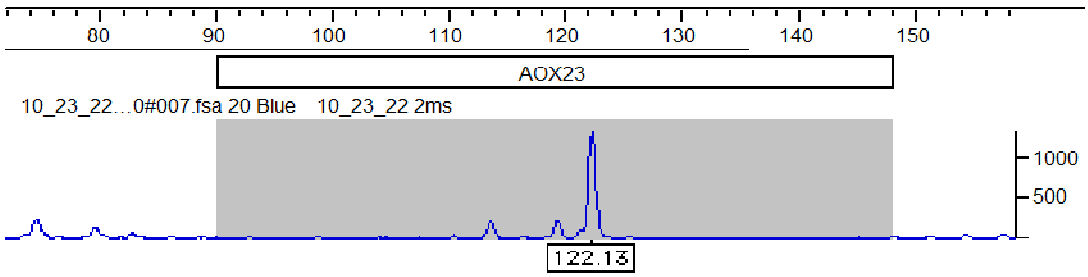


Fig. 15 d. Computer generating profile of PCR amplification products for Aox-23

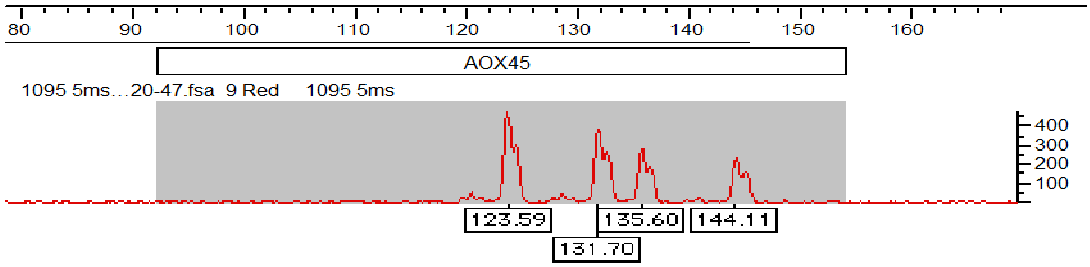


Fig. 15 e. Computer generating profile of PCR amplification products for Aox-45

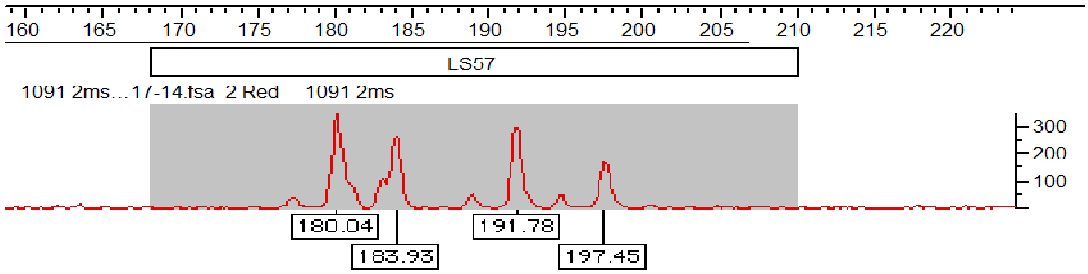


Fig. 15 f. Computer generating profile of PCR amplification products for LS-57

3. Results from the statistical analysis

A relatively high proportion of individuals could not be genotyped at the microsatellite loci. This was also the case in previous studies using the same markers (Ferguson, Suciu et al. 2000), which listed degraded DNA and the existence of null alleles as possible causes.

The two samples of adults captured at Ciotica (Black Sea coast about 10 km South from the mouth of the St. George branch) were considered only for the overall results in mtDNA and microsatellites (N=59). They were not taken into consideration for further statistical analyses because they are not migrant adults.

Three working hypotheses were considered important to be tested using statistical analyses:

1. whether there are differences between the groups undertaking spring vs. fall migration;
2. whether there are differences between YOY born in the river and having different downstream migration timing;
3. whether there has been any change in overall genetic variability in the stellate sturgeon in the period 1998/1999 to 2010.

The samples were organized in six groups according to the date of capture of groups of adults and of YOY, as follows:

- Group **"R 129"** - fall migrants (samples from 1998 and 1999captured between 10th of September and 1st of October);
- Group **"Fall7"** - possible fall migrants overwintering in the river in 2006 to 2007(captured between 1st and 16th of April 2007);
- Group **"Sprg7"** - spring migrants of year 2007 (captured between 3rd – 16th of May 2007);
- Group **"Sprg10"** - spring migrants of 2010 (captured between 24th – 29th of April);
- Group "**YOY-A**" .larger YOY captured first at the nursery ground (between 22nd and 29th of June)
- Group **"YOY-B"** - smaller YOY captured between 29th of June- 7th of July 2010.

The difference in time between these two groups of YOY stellate sturgeons with respect to arrival at rKm 123 was of 7 days.

Hardy-Weinberg conformity

The results from tests of Hardy-Weinberg conformity by locus and population are shown in Appendix Table A1. All microsatellite loci except LS-19 showed significant deviation from HW-expectations (LS-34 could not be tested due to low representation). The deviations were generally in form of heterozygote deficiencies (except for samples R128 and YOY-A at LS-19).

Linkage disequilibrium (LD)

The four microsatellite loci were tested for Linkage Disequilibrium (LD) using Genepop. No significant LD was detected in the total material or in any of the 6 subgroups.

Genic differentiation

Allele counts at the four microsatellite loci and haplotypes at the mtDNA locus are given in Appendix Table A2. There was a significant allelic differentiation between the 6 subgroups for all the genetic markers except LS-19.

Testing for null alleles at microsatellite loci

Genepop v. 4.1 can test for the existence of null alleles at microsatellite loci and their potential frequencies. The results of such analyses are found in Appendix Table A3 and show from low to very high frequencies of potential null alleles. The frequencies varied between loci and samples, but were generally high at all loci except LS-19, as shown in the table 10 below.

Table 10. Null allele estimates.

Locus	R128	Fall7	Sprg7	Sprg10	YOY-A	YOY-B
LS19	0.0000	0.0000	0.1388	0.0000	0.0000	0.0000
LS34	No inf	No inf	No inf	0.9258	No inf	No inf
LS54	0.3227	0.0000	0.3737	0.2477	0.1164	0.2101
AOX23	0.3496	0.1615	0.3399	0.3259	0.2963	0.0678

Allelelic variability at microsatellite loci

The LS34 locus stands out as extremely low in variability in all populations, while the allelic counts are rather similar between samples for the other loci in lieu of different sample sizes. Still, the YOY-A sample ranks high among them (see Table 11).

Table 11. Allele counts in microsatellites.

Locus	R128	Fall7	Sprg7	Sprg10	YOY-A	YOY-B
LS19	5	7	5	5	4	5
LS34	1	1	1	2	1	1
LS54	7	2	7	3	6	6
AOX23	6	5	9	5	5	9

Genotypic differentiation between all pairs of samples measured as R_{st} and Nei's Da were estimated by Genepop on the web. The result matrix is shown in table 12.

Table 12. Rst (below diagonal) and Da (above diagonal)

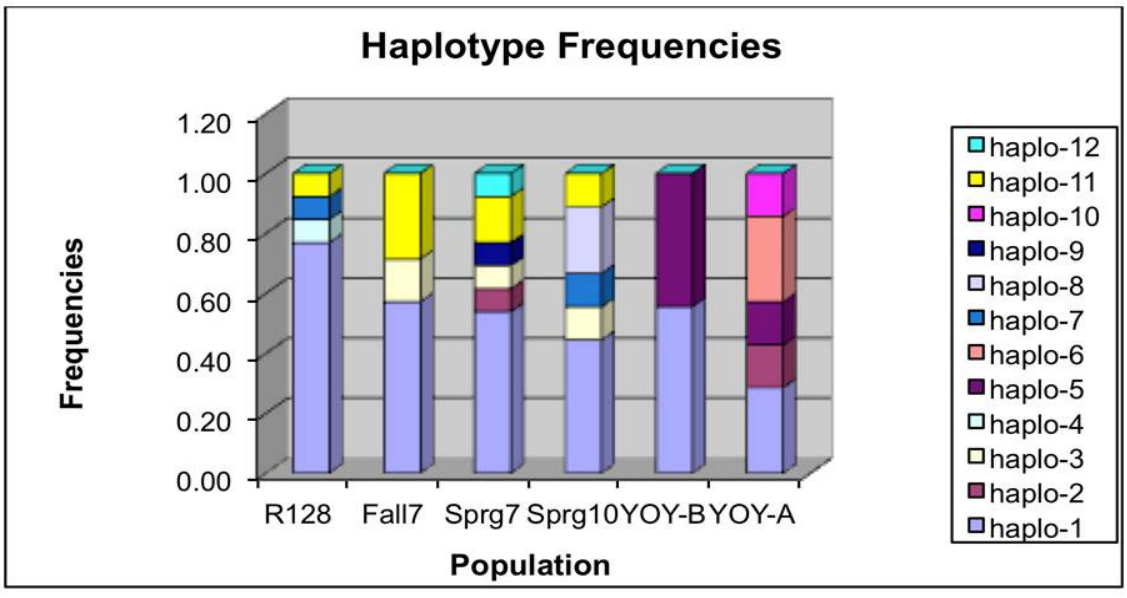
Rst / Da	R128	Fall7	Sprg7	Sprg10	YOY-A	YOY-B
R128		0.03263	0.2095	0.3000	0.6142	0.2443
Fall7	-0.1463		0.2301	0.3125	0.5822	0.2556
Sprg7	-0.0245	-0.1597		0.2756	0.5245	0.2522
Sprg10	0.1627	-0.1283	-0.1195		0.5521	0.3115
YOY-A	0.1786	-0.1195	-0.0919	-0.1190		0.4615
YOY-B	0.1770	0.0915	0.0373	0.1469	0.1325	

Fst: The prominent result in the table above is the many negative values (interpreted as nil) between population pairs. However, one of the most recent cohorts (YOY-B) show positive Fst towards the older ones as well as towards the YOY-A.

Da: The YOY-A sample shows consistently the largest genetic distance towards all other samples, and the YOY-B comes second in that respect.

Haplotype variability at mtDNA

In contrast to the microsatellites, the samples showed substantial differences with respect to variability for the gene markers at mtDNA. The results from HAPLOTYPE ANALYSIS (Haplotype analysis software) are shown in Fig. 16 and Table 13 below.



,

Fig. 16. Output from HAPLOTYPE ANALYSIS - The frequencies of haplotypes of 6 groups.

Two eye-catching properties of this graph are the high number of haplotypes found in the (small) YOY-A sample, and the declining frequency of the most common variant (haplo-1)

with time from 1998/99 to 2010. Table 13 shows details of the haplotype variability in the samples.

Table 13. Output from HAPLOTYPE ANALYSIS – INTRA-POPULATION ANALYSIS.

Population	N	A	P	N_e	R_h	H_e	D^2_{sh}
R128	13	4	1	1.641	1.615	0.423	19.538
Fall7	7	3	0	2.333	2.000	0.667	44.952
Sprg7	13	6	2	2.965	2.962	0.718	42.385
Sprg10	9	5	1	3.522	3.306	0.806	31.056
YOY-B	9	2	0	1.976	1.000	0.556	8.889
YOY-A	7	5	2	4.455	4.000	0.905	21.905
Mean	**9.667**	**4.167**	**1.000**	**2.815**	**2.480**	**0.679**	**28.121**

N: Sample size in each population A: Number of haplotypes in each population P: number of private haplotypes N_e: effective number of haplotypes, Rh: haplotype richness, He: genetic diversity, D^s_{sh}: mean genetic distance between individuals.

Cluster analysis and dendrogram construction using the DISPAN programme suite.

Cluster analyses using NJ and UPGMA algorithms gave dendrograms showing essentially the same overall topologies. Also, version using only microsatellites, only mtDNA, and both microsatellites and mtDNA combined showed essentially the same dendrogram topology.

Fig. 17 shows the NJ dendrogram based on a matrix of Nei's unbiased genetic distance (Da) including all 5 marker (4 microsatellites and one mtDNA).

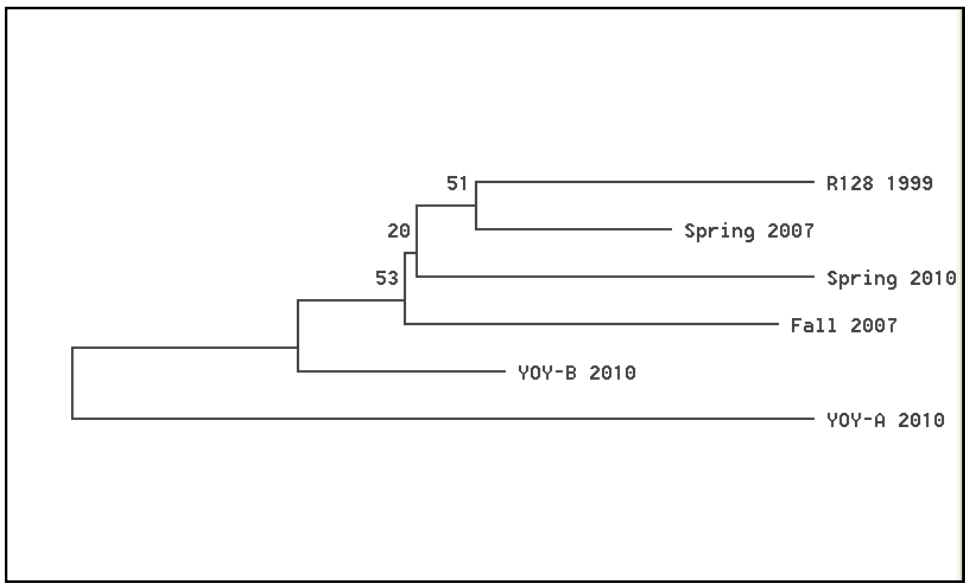


Fig. 17. NJ dendrogram (Nei's Da) based on gene frequencies at all 5 loci in all 6 samples.

The tree in Fig. 17 shows moderately high bootstrap values for bifurcations within the cluster consisting of all samples except YOY-A. The bifurcation topology within that cluster may be uncertain. However, the tree depicts effectively the significant genic differentiation between

YOY-A and others that was very evident from the analysis of genic differentiation (Appendix Table A2.) While the possible presence of null alleles as well as sample differences with respect to DNA quality cannot be ruled out as causes of false heterogeneity at the microsatellite loci (probably except LS-19), the results from the analyses of mtDNA (Appendix Table A4) offer strong support for the view that the dendrogram topology in Fig.17 probably depicts a real population differentiation.

4. Tests of working hypotheses 1-3

1. Are Fall-spawning and Spring-spawning groups genetically separated?

A test of genic differentiation (Genepop) between R128 and all the other samples revealed a mixed signal from the various loci. The P-values for the Fall / Spring differences for the different markers were: LS-19: P=0.288, LS-34: P=0.047, LS-54: P=0.005, AOX-23: P=0.154, mtDNA: P=0.348. The overall P-value for the comparisons across all loci was P=0.006, thus confirming significant genetic differences between Fall and Spring spawners. However, this P-value must be interpreted with caution since the test may be confounded: the "non-R128" group consists of several subgroups which not necessarily are from the same population. In fact, it is argumented below that e.g. that the YOY-A group is distinctly different from the YOY-B group. Also, temporal genetic changes (random genetic drift) may have taken place in the very small LDR stellate sturgeon population between 1999 and 2010. Also, it is a little worrying that the two marker with the best record with respect to genotyping score; LS-19 and mtDNA), did not show any significant genetic difference between the hypothetic Fall and Spring groups in this test.

2. Are YOY individuals born in the same year but with different downstream migration timing offspring from different populations?

The genic differentiation between YOY-A and YOY-B exclusively was explored with Genepop. The P-values for the group difference at the 5 loci were: LS-19: P=0.381, LS34: P=0.0003, LS-54: P=0.121, AOX-23: P=0.017, mtDNA: P=0.112. The overall P-value across all loci was P=0.0001. With some reservation about confounding effects by reproduction system and postnatal behavior (e.g. family-related effects) these two groups should be directly comparable as population representatives. Thus, the P-value is a strong argument for the existence of at least two genetically different YOY groups in the LDR.

3. Has there been any change in the level of genetic variability in LDR stellate sturgeon in the period 1998/1999 to 2010?

The obvious groups for comparison here are the R128 sample from 1998-1999 and the two YOY sampled in 2010. The birth dates of the 1998-1999 and 2010 samples are probably separated by 15-20 years. Since YOY-A and YOY-B have been shown to be genetically different (cf. point 2 above), separate comparisons with R128 were performed. The relevant population parameters for testing were the ranks of gene diversity among individual within groups (1-Qinter) per locus and averaged over loci (Genepop option 5.1). The results are tabulated below (Table 14) (for LS-34 no statistics could be calculated due to low genotype scoring).

Table 14. The gene diversity among individuals within groups.

	LS-19	LS-54	AOX-23	mtDNA	Mean all loci (>=2 ind.)
R128	0.700	0.8036	0.9333	0.4231	0.5803
YOY-A	0.6181	0.8810	0.9028	0.5556	0.6846
YOY-B	0.7667	0.8668	0.7143	0.9048	0.6727

The gene diversity ranking of the three test groups vary between loci in an inconsistent way, but nominally the two YOY groups come out on average "better" than the sample from 1998-1999. With due reservations as to the accuracy of this analysis (which can be compromised by many factors, e.g. small samples, null alleles, low scoring), these gene diversity estimates do not indicate that the genetic variability per locus has declined in the period spanned by these temporal samples.

Discussion

The present study encountered some issues related to sampling procedure, tissue quality and the robustness of genetic results.

1. The sampling in the present study was, for reasons of shear availability, not optimal. To a large degree the project had to use what was available, as described in Materials and Method. It is assumed, though, that the present results will enable a more directed and focused sampling design in future studies.
2. The tissues and varying quality of extracted DNA (from high molecular weight to heavily degraded) affected the reactions and subsequent analyses. Although the DNA concentration was set to 100 ng/µl working solution, due to the DNA degradation different PCR products concentration were obtained. These were observed in horizontal electrophoresis of digested fragments within RFLP analyses (Fig. 13 and 14). Therefore, adequate routines to ensure high quality DNA for analysis must have high priority in the planning of future studies.
3. Suboptimal primers for microsatellite DNA amplification may have contributed to the low success rate for microsatellite genotyping. The primers were previously developed for other sturgeon species and gave, except the LS19, very low scoring success and were suggested by the adequate software to be ridden by high frequencies of null alleles. There is an urgent need for developing new primers for microsatellites loci which are specific for the stellate sturgeon, for use in future studies.
4. Due to number of causes (cf. pts 1-3 above), the number of individuals for analysis became very small in this study. This affected the level of detail that could be aimed at in the analysis, and the possibility of obtaining statistically significant results for some of the contrasts performed.

Despite these different issues, several important results were obtained in this study. Thus, a panel of microsatellite and mtDNA genetic markers, which rely on non-invasive sampling techniques (e.g. fin clips), have been tested out on Acipenser *stellatus* in the Lower Danube river. They open opportunities for investigating if reproductive units / sub-populations of the

LDR and NW Black Sea stellate sturgeons exist. The levels of polymorphism appear sufficient for ensuring effectiveness of such molecular tools.
Probably due to the causes in points 2-4 above, the single samples were often far out of Hardy-Weinberg equilibrium, mostly in terms of heterozygote deficiencies. However, some of the deficiency might be Wahlund effect due to physical mixture of specimens from populations with different gene frequencies.

In spite of all the potential anomalies of the microsatellites, there are some observations that probably can be acknowledged with confidence because the anomalies would be expected to have affected the loci and the samples equally. Thus, the present results indicate that the actual genetic variability in the LDR stellate sturgeon has not declined appreciably after the moratorium was implemented. It was shown that the level was not altered between the birth year of the 1998-1999 samples and the young of the year samples in 2010, which actually spans 15-20 years.

In contrast to the suboptimal performance of some of the microsatellites, the mtDNA marker showed a consistently high scoring efficiency. A very significant result from the haplotype analyses was the demonstration of a very significant differentiation between YOY-A and YOY-B, two samples taken on nearby locations in 2010 (see Case study YOY below). This discovery is promising for the conservation of LDR river stocks, and will be very important for the design of future experiments to shed light on the real stock structure, and ultimately for the implementation of a sustainable management of these endangered creatures.

Case study YOY

According to their body sizes, the YOY-A and YOY-B might be the result of at least two different / successive spawning events. This project revealed genetic data in support of that.
The data from 2010 on natural spawning of stellate sturgeons could be compared with the previous ones, from 2008 and 2004 (Suciu, Onara et al. 2011). Monitoring the YOY at rKm 123 on the Danube River, the same patterns were observed (see Fig 18 and 19) at least two groups of YOY were monitored at the feeding ground at rKm 123 in year 2008 and even three groups can be observed in 2004. These previous and current knowledge based on genetic data of YOY of 2010 revealed that there are at least two spawning events according to the two or three groups of YOY arriving at the nursery (feeding) ground at rKm 123.

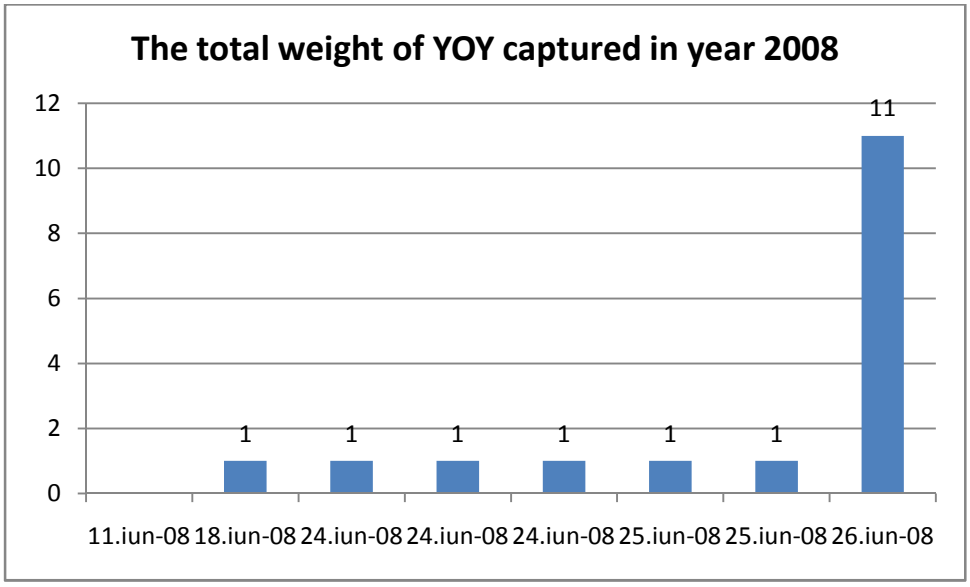


Fig 18. Total weight (TW) of YOY Stellate sturgeons captured at rKm 132 (N=8) in year 2008 (Suciu, Onara et al. 2011)

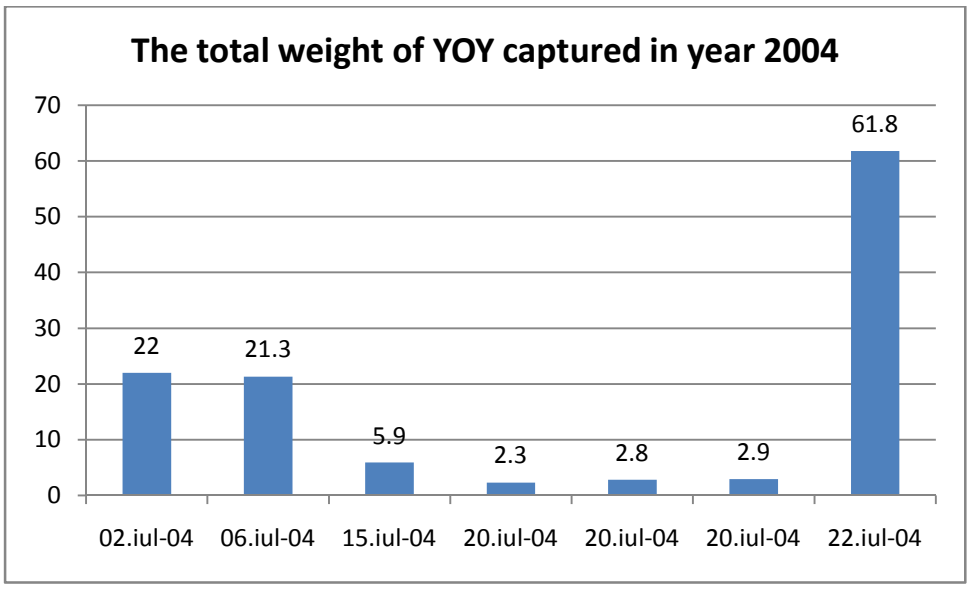


Fig 19. Total weight (TW) of YOY stellate sturgeons sampled at the rKm 123 (N=7) in year 2004 (Suciu, Onara et al. 2011)

The fact that at least two spawning events take place in the Lower Danube river is confirmed by the monthly distribution of captures of stellate sturgeons in the Lower Danube River, recorded by the Romanian Fishery Administration (FA) in year 2004 and 2005, in the Romanian sector of Danube River upstream Prut River mouth (rKm 135) shown in fig. 20 (Sturgeons of Romania and CITES).

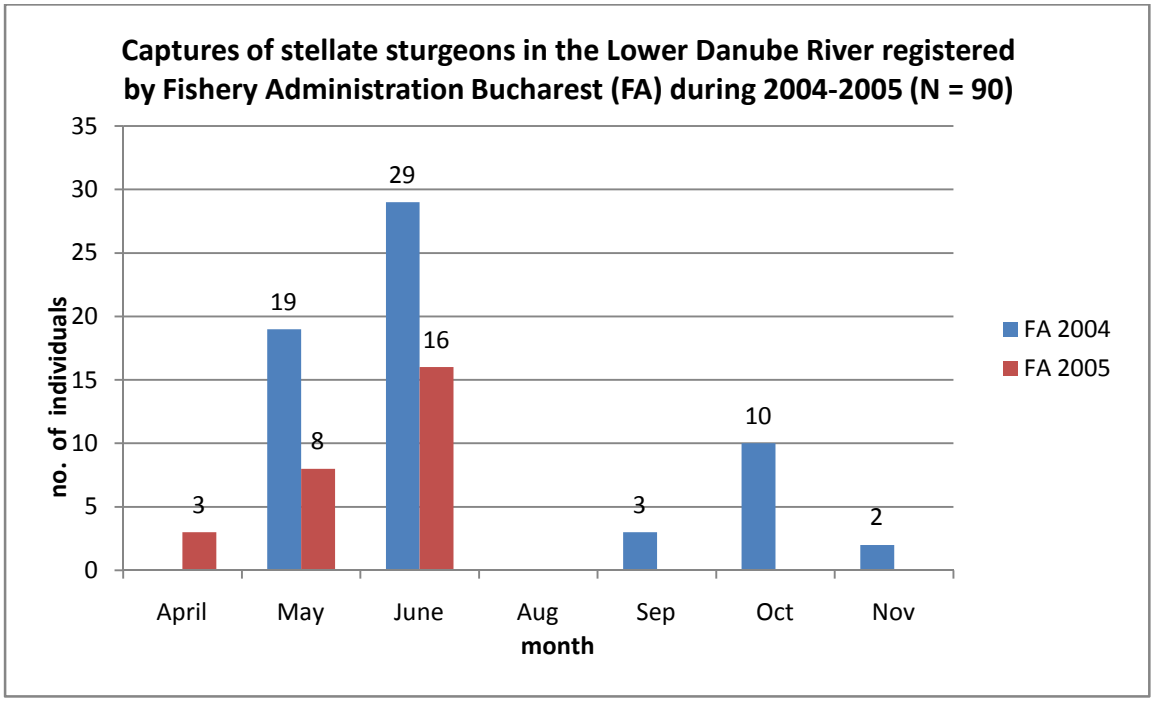


Fig. 20. The total number of captures of stellate sturgeons registered upstream Prut River mouth (rKm 135) in years 2004 and 2005 (Romanian sturgeons and CITES – Captures -FA).

Figure 20 shows that the migrating adult stellate sturgeons were captured in important numbers in the upper LDR in May and June. That means that a group of females have undertaken spawning migration even in June and their offspring could be found in the river in July -August.

In year 1997 (Suciu, Baboianu et al. 1997) during a study about distribution of YOY sturgeons upstream Tulcea and on St George branch of Danube the SRG team involved in the study, captured one young stellate sturgeon of 20 cm total length (TL) (Fig 21) during 23rd - 24th of July at Danube rKm 310.5, 187.5 Km upstream the feeding ground where YOY are monitored since year 2000. These findings enforced the data from Romanian Fishery Administration showing that stellate sturgeons come for spawning even in June.

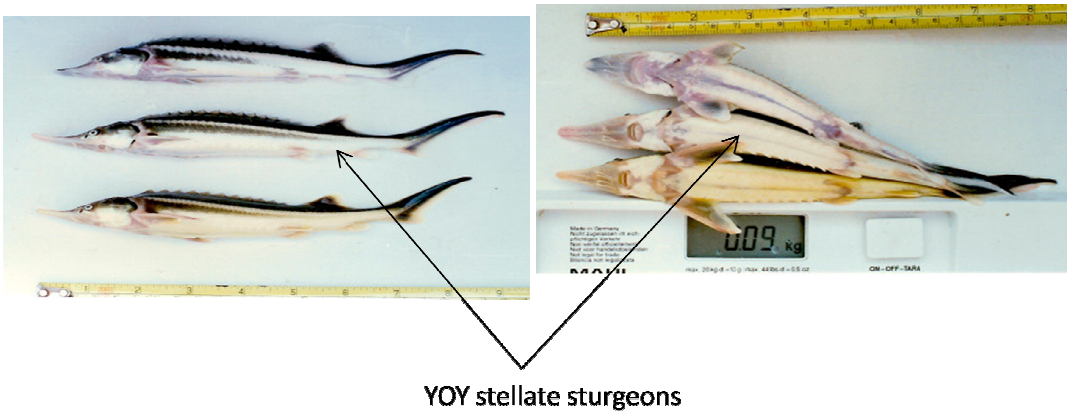


Fig. 21 Lateral and ventral view of sturgeons YOY captured at Danube rKm 310.5 (Suciu, Baboianu et al. 1997)

YOY – A group

Statistic analyses revealed that YOY-A group is special with respect to genetic variability. YOY-A has two most "private" alleles despite its small size, and are also superior in other parameters such as: effective number of haplotypes, haplotypic richness and genetic diversity (Table 13, A6 and Fig. 16).

The NJ and UPGMA trees dendrograms as well as the other statistic analyses results indicated that YOY - A are the offspring / recruits of a real sub-population, which, due to exceptionally high water levels of the Danube River in spring and summer of year 2010, escaped illegal fishing still present at a certain level in the whole river, and spawned in the upper part of the LDR. This seems to have been a quite rare event during the last ten years.

The difference between YOY-A and the other groups ("other groups" corresponds the other cluster in the dendrogram – see Fig. 16) was significant also for mtDNA (Fig. A5) which has a satisfactory high scoring in the materials.

.

Conclusions

The present study has offered supporting evidence for the existence of a population substructuring within the Lower Danube River basin stellate sturgeon, even though the number of individuals sampled were low. The genetic signal is not univocal, and the real nature of the population substructure should await further study. The YOY groups from 2010 stood out as genetically different from earlier cohorts. This was evident from both types of markers (microsatellites and mtDNA). The presently demonstrated evidence of structuring is sufficiently suggestive to make further studies worth pursuing.

A development of a much more comprehensive set of genetic markers specially developed for *A. stellatus*, for use in future and more accurate and comprehensive studies on the stellate sturgeon population structure is recommended.

Despite the illegal fishing which still exists in the Lower Danube River, 10 years of fishing moratorium might have had a conserving effect on genetic variability, as indicated by the similar level of genetic variability in samples spanning more than a decade (1998 and 2010). In fact, the small (N=7) YOY-A sample ranked highest in number of different haplotypes in the materials. There might be a possibility that YOY-A are offspring from artificial breeding, and as such is characterized by a breeding regime which is designed to create high heterozygosity. Alternatively, the moratorium could have allowed a larger number of parents to take part in the spawning and thus enhanced the genetic variability in the most recent cohorts. A future study in about 5 years would be necessary to assess whether the moratorium has been successful indeed with respect to conserving the genetic variability.

During this study could not be effectively assess whether the Romanian Supportive stocking programme have had wanted or unwanted effects due to the facts that first juvenile of stellate sturgeons were stocked in the Danube in 2005 and the long life cycle of stellate sturgeons

which first undertake spawning migration when become mature at about 5 – 6 years (males) and 7-10 years (females). Further studies from 2011 and on are required to assess the Supportive Stocking Programme effectiveness.

The present study was undertaken on a small number of individuals from small groups of stellate sturgeons in the Lower Danube River. It was not designed for detailed population genetic analysis, as this would not have been feasible within the resources available.

Studies based on larger number of individuals from known subpopulations are required to elucidate details of a population structure. To achieve this, identification of natural spawning areas of stellate sturgeons using ultrasonic telemetry techniques and adequate sampling gear/techniques for eggs and larvae are necessary. Adults from both fall and spring migrating races are captured and tagged with acoustic transmitters and their movements monitored by active telemetry and / or placing acoustic receiver arrays in the potential spawning sites in order to understand the spawning behaviour and to identify particular spawning areas. Fin clip samples should be collected from each captured individual, and the DNA extracted and used in further analyses of a substantially enhanced panel of mtDNA and nuclear markers.

Also, the degree of interaction (migrations, spawning) between stellatus population inhabiting the Black Sea and Azov Sea should be assessed by systematic, large international tagging experiments. Such knowledge is crucial if the mangement goal is to preserve species and genetic variability / resources within species.

Acknowledgments

My sincere gratitude goes to my supervisor Professor Jarle Mork for his valuable guidance and support during the whole process of this study. I will always be thankful for his huge help during my first steps in the population genetics field and with his vast knowledge and enthusiasm has awaken in me a great interest for this exciting scientific field. He has been encouraging, motivating and helping me to get the most of this study.

I am very grateful to Dr. Radu Suciu the head of the Sturgeons Research Group within the Danube Delta National Institute for giving me the opportunity to undertake this study and for providing me valuable data about the ecology and life history of stellate sturgeon population in the Lower Danube River.

I am sincerely thankful to my co-supervisors Dr. Dalia Onara from the Danube Delta National Institute and Professor Marieta Costache from the University of Bucharest for their guidance and logistic support, during the lab work in Romania.

I am highly indebted to Innovation Norway and Romanian Government for providing the scholarship that enables me to study in the most beautiful place on Earth, Trondheim.

My heartfelt thanks go to the two greatest ladies within NTNU Department of Biology, Lisbeth Aune and Marian Forde who guided my first steps while studying in Norway.

I also want to thank to my colleagues of the molecular biology labs in Tulcea and Bucharest, Lili, Andrea and Sergiu, for helping me in the lab and to Marian I. and Marian P. who have helped me during the field work of capturing YOY in year 2010.

My deepest love goes to my family, my husband and my daughter, whose love and support was sometimes the only thing that has helped me keep going.

All my thankfulness goes to my mum and dad who have taken care of my daughter while I have been studying in Norway and also during the field and lab work in Romania.

Many thanks to my friends forever, Raluca and June, who offered me moral support and pertinent advices when needed.

Finally, I would like to thank to all my colleagues under MaCoDev programme, it was a pleasure and a nice experience for me to study in that great international environment.

Bibliography

Antipa, G. (1909). Ichthyological Fauna of Romania (in Romanian), Institutul De Arte Grafice "Carol Göbl" Bucuresti: 251-254.

Artyukhin, Y. P. (1979). *Контактные задачи для круглых пластин и сферических оболочек* Issled. Teor. Plastin i Obolochek, 14 Kazan University, Kazan: 123-139.

Bacalbasa-Dobrovici, N. (1991). Statut des différentes espèces d'esturgeons dans le Danube roumain : problèmes liés à leur maintenance. Premier colloque international sur l'esturgeon,. W. Patrick. Bordeaux, , Editions Cemagref-Dicova,185-192.

Bacalbaşa-Dobrovici, N. (1997). "Endangered migratory sturgeons of the lower Danube River and its delta." Environmental Biology of Fishes **48**(1): 201-207.

Bacalbasa - Dobrovici, N. and N. Patriche (1999). "Environmental studies and recovery actions for sturgeon in the Lower Danube River system." Journal of Applied Ichthyology **15**(4-5): 114-115.

Bănărescu, P. (1964). Pisces-Osteichthyes. Fauna of People Republic of Romania (in Romanian) Editura Acad. Republicii Populare Romîne, Bucuresti.

Billard, R. and G. Lecointre (2000). "Biology and conservation of sturgeon and paddlefish." Reviews in Fish Biology and Fisheries **10**(4): 355-392.

Bloesch, J. (2006). The ultimate need for the implementation of sturgeon protection in the Danube River Basin - a view of 2006 and call for actions with the Sturgeon Action Plan under the Bern Convention. IAD Limnological Reports, 36.

Bloesch, J., T. Jones, et al. (2006). Action Plan for the conservation of Sturgeons (Acipenseridae) in the Danube River Basin, Council of Europe.

Ciolac, A. and N. Patriche (2005). "Biological aspects of main marine migratory. Sturgeons in Romanian Danube River. Migration of fishes in Romanian Danube River." Applied Ecology and Environmental Research **Res. 2 (1)**: 143-163.

Doukakis, P., D. Erickson, et al. (2008). Field and Genetic Approaches to Enhance Knowledge of Ural River Sturgeon Biology. Rescue of Sturgeon Species in the Ural River Basin. V. Lagutov, Springer Netherlands**:** 277-292.

Dudu, A., S. E. Georgescu, et al. (2008). "Microsatellites DNA variation in the Black Sea stellate sturgeon, *Acipenser stellatus* " Lucrări stiintifice Zootehnie si Biotehnologii **Vol. 41: 78-82**.

Eliades, N. G. and D. G. Eliades (2009). “Haplotype Analysis: Software for Analysis of Haplotype Data”. Distributed by the authors. Forest Genetics and Forest Tree Breeding, Georg-Augst University Goettingen, Germany.

Erickson, D. L., J. A. North, et al. (2002). "Movement and habitat use of green sturgeon Acipenser medirostris in the Rogue River, Oregon, USA." Journal of Applied Ichthyology **18**(4-6): 565-569.

Ferguson, A., R. Suciu, et al. (2000). Genetic population structure of endangered sturgeon species of Lower Danube. Royal Society Joint Projects with Central / Eastern Europe and the former Soviet Union, Final report, August 2000**:** 15 p.

Glaubitz, J. C. (2004). "convert: A user-friendly program to reformat diploid genotypic data for commonly used population genetic software packages." Molecular Ecology Notes **4**(2): 309-310.

Guti, G. (2006). Past and present status of sturgeons in Hungary. Proceedings 36th International Conference of IAD. Austrian Committee Danube Research / IAD Vienna:143-147.

Hallerman, E., Ed. (2003). "Population genetics: Principles and Applications for Fisheries Scientists", American Fisheries Society, Bethesda, Maryland, 458pp.

Hensel, K. and J. Holčík (1997). "Past and current status of sturgeons in the upper and middle Danube River." Environmental Biology of Fishes **48**(1): 184-200.

Ivanov, A. P. (1987). Fishing in natural waters (in Russian), Agropronizdat, Moskva: 420.

Kieffer, M. and B. Kynard (2004). Pre-spawning Migration and Spawning of Connecticut River Shortnose Sturgeon (manuscript).

Knight, C., T. Kristensen, et al. (2009). Development of a management strategy for Danube sturgeon: A case study with beluga (Huso huso) (unpublish article).

Kynard, B. and M. Horgan (2002). "Ontogenetic behavior and migration of Atlantic sturgeon, *Acipenser oxyrinchus oxyrinchus*, and shortnose sturgeon, *A. brevirostrum*, with notes on social behavior." Environmental Biology of Fishes **63**(2): 137-150.

Kynard, B., R. Suciu, et al. (2002). "Migration and habitats of diadromous Danube River sturgeons in Romania: 1998–2000." Journal of Applied Ichthyology **18**(4-6): 529-535.

Lenhardt, M., I. Jarić, et al. (2008). The Key Threats to Sturgeons and Measures for Their Protection in the Lower Danube Region. Rescue of Sturgeon Species in the Ural River Basin. V. Lagutov, Springer Netherlands**:** 87-96.

Lucas, M. C. and E. Baras (2001). Migration of fresh water fish, Blackwell Science:2-6.

Ludwig, A. (2008). "Identification of Acipenseriformes species in trade." Journal of applied ichthyology **24**: 2-19.

Ludwig, A., L. Debus, et al. (2002). "A Molecular Approach to Control the International Trade in Black Caviar." International Review of Hydrobiology **87**(5-6): 661-674.

Manea G. (1966). Contribution to the study on sturgeons in Romanian waters and their reproduction in relation to the hydroenergetic construction in the Lower Danube river. Aspects of the sturgeons biology. (in Romanian). Buletinul Institutului de Cercetari si Proiectari Piscicole **25: 62-86**.

May, B., C. C. Krueger, et al. (1997). "Genetic variation at microsatellite loci in lake sturgeon: primer sequence homology in Acipenser and Scaphirhynchus." Can. J. Fish. Aquat. Sci. **54**: 1542-1547.

Navodaru, I., M. Staras, et al. (1999). Management of the sturgeon stocks of the Lower Danube River system. . The Delta's: state-of-the-art protection and management. Conference proceedings, Tulcea, Romania: 229-237.

Onara, D., M. Paraschiv, et al. (2007). Management applications of genetic structure of sturgeon populations in the lower Danube River, Romania. Book of abstracts of the 12th European Congress of Ichthyology: 9 – 13 Sept. 2007, Cavtat (Dubrovnik), Croatia. Pp 207.

Paraschiv, M., R. Suciu, et al. (2006). "Present state of sturgeon stocks in the Lower Danube River, Romania." Austrian Committee Danube Research / IAD, Vienna: 152-158 pp.

Pavlov, D. S. (1989). Structures assisting the migrations of non-salmonid fish: USSR. Food and Agriculture Organization of the United Nations Rome. FAO Fisheries Technical Papers **(308):** 8-9.

Prokes, M., V. Barus, et al. (1996). "On ventral head black pigmentation in the *Acipenser stellatus* Pallas, 1771." Acta Universitatis Carolinae Biologica **40: 171-175**.

Reinartz, R. (2002). Sturgeons in the Danube River. Bilogy, Status, Conservation. Literature study, International Association for Danube Research (IAD). Bezirk Oberpfalz, Landesfichereiverband Bayem, e.V: 118-131.

Reinartz, R. (2002). Sturgeons in the Danube River. Bilogy, Status, Conservation. Literature study, International Association for Danube Research (IAD). Bezirk Oberpfalz, Landesfichereiverband Bayem, e.V: 118-131.

Shubina, T., A. Popova, et al. (1989). *Acipenser stellatus* Pallas, 1771. The Freshwaterfishes of Europe. General introduction to Fishes Acipenseriformes. J. Holcik, AULA-Verlag Wiesbaden: 394-434. **Vol.1 Part II:** 394-434.

Stabile, J., J. R. Waldman, et al. (1996). "Stock Structure and Homing Fidelity in Gulf of Mexico Sturgeon (Acipenser Oxyrinchus Desotoi) Based on Restriction Fragment Length Polymorphism and Sequence Analyses of Mitochondrial DNA." Genetics **144(2): 767-775**.

Suciu, R. (2008). Sturgeons of the NW Black Sea and the Lower Danube River Countries. International Expert Workshop on CITES Non-Detriment Findings Cancun / Mexico, November 17th-22nd, 2008.

Suciu, R., C. Baboianu, et al. (1997). Protection and recovery of migratory sturgeon populations in the Lower Danube River (in Romanian). Tulcea, Romania, Danube Delta National Institute**:** 27.

Suciu, R., D. Onara, et al. (2011). Contributions to Understanding the Spawning Ecology of Beluga Sturgeons in the Lower Danube River. International Conference on Conservation, Recovery and Sustainable Use of Danube River Sturgeons, 29th of March - 2nd of April 2011, Tulcea, Romania.

Suciu, R., M. Paraschiv, et al. (2007). Behaviour of early life stages (ELS) of sturgeons in the Danube River. Interim report. Conservation and sustainable use of Sturgeons of the Lower Danube river - Project funded by NASR, BIOTECH Programme. Contract No. 120 / 2006.

Suciu, R., M. Paraschiv, et al. (2007). Romanian Supportive Stocking Program of the Danube River with endangered species of sturgeons XVI - Annual Symposium of Danube Delta Institute (20 -21 Sept. 2007), Tulcea, Romania.

Suciu, R., P. Prodohl, et al. (2001). Genetic variation in sturgeon species of the lower Danube River. Abstract of 10th European Congress of Ichthyology, Prague.

Suciu, R., C. Rosten, et al. (2011). Downstream movement of YOY beluga sturgeons in the Lower Danube River. International Conference on Conservation, Recovery and Sustainable use of Danube River Sturgeons 29 th March – 2nd April 2011, Tulcea, Romania.

Vassilev, M. (2006). Lower Danube - the last refuge for surviving of sturgeon fishes in the Black Sea Region. Conference Proceedings, Balwois, Ohrid, Macedonia

Vecsei, P., D. Peterson, et al. (2007). "Threatened fishes of the world, Acipenser stellatus Pallas, 1771(Acipenseridae)." Environmental Biology of Fishes **78**(3): 211-212.

Wang, Y., F. Binkowski, et al. (1985). "Effect of temperature on early development of white and lake sturgeon, Acipenser transmontanus and A. fulvescens." Environmental Biology of Fishes **14**(1): 43-50.

Wirgin, I. I., J. E. Stabile, et al. (1997). "Molecular analysis in the conservation of sturgeons and paddlefish." Environmental Biology of Fishes **48**(1): 385-398.

Websites:

Convert website *** http://www.agriculture.purdue.edu/fnr/html/faculty/rhodes/students%20and%20staff/glaubitz/software.htm

Genelink *** http://www.genelink.com

"Genepop on the web"*** http://genepop.curtin.edu.au/

Genepop 4.1 *** http://kimura.univ-montp2.fr/~rousset/Genepop.htm.

Dispan *** http://iubio.bio.indiana.edu/soft/molbio/ibmpc/dispan.readme

Fstat website *** http://www2.unil.ch/popgen/software/fstat.htm

Haplotype analysis software *** http://haplotypeanalysis.wordpress.com/software/

IUCN Red List*** http://www.iucnredlist.org/apps/redlist/details/229/0

NEBcutter*** http://tools.neb.com/NEBcutter2/

Romanian sturgeons and CITES – Captures –FA *** http://ddni.ro/rosturgeons/index.php?option=com_content&task=view&id=29&Itemid=43

Romanian Sturgeons and CITES website-fishing moratorium*** http://ddni.ro/rosturgeons/images/documente/joint_ministerial_order_on_sturgeon_conservation_inro.pdf

Wikipedia*** http://en.wikipedia.org/wiki/File:Danubemap.jpg

WWF - Action Plan for sturgeons*** http://assets.panda.org/downloads/sturgeon_action_plan_brochure.pdf

Appendix

Table A1. Genepop Option 1.3 (Hardy-Weinberg probability tests) output results for each microsatellite locus in each subgroup.

```
Estimation of exact P-Values by the Markov chain method.
Markov chain parameters for all tests:
Dememorization:1000 Batches:100 Iterations per batch:1000

Estimation of exact P-Values by the Markov chain method.
-----------------------------------------
Markov chain parameters for all tests:
Dememorization:              1000
Batches:                     100
Iterations per batch:        1000
Hardy Weinberg: Probability test


==========================================
     Results by locus
==========================================

Locus "LS19"
-----------------------------------------------------------
                             Fis estimates
                            ---------------
POP          P-val   S.E.    W&C     R&H     Steps
---------- ------- ------- ------- ------- ------
R128       0.4317  0.0103 -0.3636 -0.2083  12225 switches
Fall7      0.5420  0.0195  0.1193  0.0448   3532 switches
Sprg7      0.1876  0.0074  0.2222  0.2923  13255 switches
Sprg10     0.7778  0.0067 -0.0312 -0.0242  12076 switches
YOY-B      0.2751  0.0127  0.1011 -0.0099   5960 switches
YOY-A      1.0000  0.0000 -0.0870 -0.0833  15895 switches
-----------------------------------------------------------
All (Fisher's method):
Chi2:    9.3349
Df   :    12.0000
Prob :    0.6741


Locus "LS34"
----------------------------------------
                             Fis estimates
                            ---------------
POP          P-val   S.E.    W&C     R&H     Steps
---------- ------- ------- ------- ------- ------
R128          -
Fall7         -
Sprg7         -
Sprg109       -
YOY-B         -
YOY-A         -
-------------------------------------------------

Locus "LS54"
-----------------------------------------------------------
                             Fis estimates
```

			Fis estimates		
POP	P-val	S.E.	W&C	R&H	Steps
R128	0.2704	0.0143	0.2727	0.1194	4776 switches
Fall7	1.0000	0.0000	0.0000	0.0000	5573 switches
Sprg7	0.0049	0.0020	0.6000	0.5000	3221 switches
Sprg10	0.0250	0.0017	0.7500	0.5400	18699 switches
YOY-B	0.0097	0.0024	0.5135	0.5167	6797 switches
YOY-A	00647	0.0055	0.2308	0.1000	5254 switches

All (Fisher's method):
Chi2: 35.3850
Df : 12.0000
Prob : 0.0004

Locus "AOX23"

			Fis estimates		
POP	P-val	S.E.	W&C	R&H	Steps
R128	0.0010	0.0009	0.8214	0.7600	5665 switches
Fall7	0.0218	0.0039	0.5294	0.3125	6717 switches
Sprg7	0.0000	0.0000	0.6667	0.4074	3154 switches
Sprg10	0.0002	0.0001	0.8077	0.6500	8370 switches
YOY-B	0.0434	0.0112	0.2615	0.1245	2781 switches
YOY-A	0.0028	0.0013	0.8000	0.6250	5682 switches

All (Fisher's method):
Chi2: Infinity
Df : 12.0000
Prob : High. sign.

Table A2.Output from Genepop option 3.1: Genic differentiation at all loci in all populations.

```
---------------------------------------------------------------------------
Number of populations detected : 6
Number of loci detected        : 5
Markov chain parameters
Dememorisation       : 1000
Batches              : 100
Iterations per batch : 1000


Locus: LS19
===========
Pop       Alleles:
-------------------------------------------------------------
          115  121  124  127  130  133  136  139  145    Total

R128       0    0    0    1    11   4    3    3    0       22
Fall7      1    1    1    0    6    1    5    1    0       16
Sprg7      2    0    0    0    9    4    8    0    1       24
Spr10      0    0    1    1    10   6    6    0    0       24
YOY-A      0    0    0    0    4    4    3    0    1       12
YOY-B      0    0    1    0    11   3    2    1    0       18

Total:     3    1    3    2    51   22   27   5    2       116
P-value = 0.46559    S.E. = 0.0180167 (22532 switches)


Locus: LS34
===========
Pop       Alleles:
-------------------------------------------------------------
          144  147    Total

R128       0    18      18
Fall7      0    4       4
Sprg7      0    2       2
Spr10      1    13      14
YOY-A      6    0       6
YOY-B      0    8       8

Total:     7    45      52
P-value = 0    S.E. = 0 (39784 switches)


Locus: LS54
===========
Pop       Alleles:
-------------------------------------------------------------
          161  165  169  173  177  181  185  189  193    Total

R128       0    1    0    1    1    1    1    6    5       16
Fall7      0    0    0    0    0    1    1    0    0       2
Sprg7      0    2    1    2    1    1    1    0    2       10
Spr10      0    5    0    0    0    6    0    1    0       12
YOY-A      4    1    3    0    0    0    2    1    1       12
YOY-B      0    4    2    0    1    2    4    1    0       14
-------------------------------------------------------------
Total:     4    13   6    3    3    11   9    9    8       66
P-value = 0    S.E. = 0 (26169 switches)
```

Locus: AOX23

```
============
Pop       Alleles:
-------------------------------------------------------------------------------
-------------------
          92   95   98   101  110  113  116  119  122  125  128  131  134
137  140  146 Total

R128      0    4    0    2    0    2    1    1    0    0    2    0    0
0    0    0      12
Fall7     0    4    3    0    0    0    0    0    1    0    0    1    1
0    0    0      10
Sprg7_14  0    6    4    3    0    2    0    1    0    1    0    1    0
0    1    1      20
Sprg10_   0    0    4    4    1    0    2    0    0    1    0    0    0
0    0    0      12
YOYA_3    2    0    8    0    0    0    0    0    2    0    1    1    0
0    0    0      14
YOYB_7    0    3    1    1    0    1    0    0    5    2    3    1    0
1    0    0      18
-------------------------------------------------------------------------------
-------------------
Total:    2    17   20   10   1    5    3    2    8    4    6    4    1
1    1    1      86
P-value = 0    S.E. = 0 (15406 switches)
```

Locus: mtDNA

```
============
Pop       Alleles:
-------------------------------------------------------------------------------
          1   2   3   4   5   6   7   8   9   10  11  12    Total

R128      10  0   0   1   0   0   1   0   0   0   1   0      13
Fall7     4   0   1   0   0   0   0   0   0   0   2   0      7
Sprg7     7   1   1   0   0   0   0   0   1   0   2   1      13
Spr10     4   0   1   0   0   0   1   2   0   0   1   0      9
YOY-A     2   1   0   0   1   2   0   0   0   1   0   0      7
YOY-B     5   0   0   0   4   0   0   0   0   0   0   0      9

Total:    32  2   3   1   5   2   2   2   1   1   6   1      58
P-value = 0.02806    S.E. = 0.00804702 (10603 switches)
```

```
===============================================================================
```

P-value across all loci

```
(Fisher's method)
-----------------------------------------------
Locus          P-Value
-------------  --------
LS19           0.46559
LS34           0
LS54           0
AOX23          0
mtDNA          0.02806
```

All: Chi2= Infinity (df= 10), highly significant
Normal ending.

Table A3. Results from Genepop v. 4.1 test for the existence and frequencies of null alleles in the present microsat. materials.

(Locus by population) table of estimated null allele frequencies

Locus:	Populations					
	R128	Fall7	Sprg7	Spr10	YOY-A	YOY-B
LS19	0.0000	0.0000	0.1388	0.0000	0.0000	0.0000
LS34	No inf	No inf	No inf	0.9258	No inf	No inf
LS54	0.3727	0.0000	0.3737	0.2477	0.1164	0.2101
AOX23	0.3496	0.1615	0.3399	0.3259	0.2963	0.0678

Table A4. Output from Genepop option 1.3: Genic differentiation at mt DNA in all populations.

Number of populations detected : 6

Number of loci detected : 1

Markov chain parameters

Dememorisation : 10000

Batches : 100

Iterations per batch : 5000

Locus: mtDNA

Pop	Alleles:												
	1	2	3	4	5	6	7	8	9	10	11	12	Total
R128	10	0	0	1	0	0	1	0	0	0	1	0	13
Fall7	4	0	1	0	0	0	0	0	0	0	2	0	7
Sprg7	7	1	1	0	0	0	0	0	1	0	2	1	13
Spr10	4	0	1	0	0	0	1	2	0	0	1	0	9
YOY-A	2	1	0	0	1	2	0	0	0	1	0	0	7
YOY-B	5	0	0	0	4	0	0	0	0	0	0	0	9
Total:	32	2	3	1	5	2	2	2	1	1	6	1	58

Locus	Population pair	P-Value	S.E.	Switches
mtDNA	Fall7& R128	0.57419	0.00206	158364
mtDNA	Sprg7& R128	0.50780	0.00315	163270
mtDNA	Sprg7& Fall7	1.00000	0.00000	160074
mtDNA	Spr10& R128	0.54572	0.00222	173301
mtDNA	Spr10& Fall7	0.77963	0.00143	193417
mtDNA	Spr10& Sprg7	0.63622	0.00235	161234
mtDNA	YOY-A& R128	0.02494	0.00108	135119
mtDNA	YOY-A& Fall7	0.13488	0.00141	157056
mtDNA	YOY-A& Sprg7	0.18847	0.00257	141988
mtDNA	YOY-A& Spr10	0.15264	0.00184	147567
mtDNA	YOY-B& R128	0.01651	0.00068	176109
mtDNA	YOY-B& Fall7	0.05605	0.00090	206098
mtDNA	YOY-B& Sprg7	0.05164	0.00111	163906
mtDNA	YOY-B& Spr10	0.05420	0.00123	177186
mtDNA	YOY-B& YOY-A	0.10960	0.00138	189910

P-value for each population pair across all loci

(Fisher's method)

Population pair	Chi2	df	P-Value
R128& Fall7	1.10958	2	0.574192
R128 & Sprg7	1.355319	2	0.507804
Fall7& Sprg7	0.000000	2	1.000000
R128 & Spr10	1.211284	2	0.545724
Fall7& Spr10	0.497882	2	0.779626
Sprg7& Spr10	0.904415	2	0.636222
R128& YOY-A	7.382885	2	0.024936
Fall7& YOY-A	4.006740	2	0.134880
Sprg7& YOY-A	3.337612	2	0.188472
Spr10& YOY-A	3.759294	2	0.152644
R128& YOY-B	8.207336	2	0.016512
Fall7& YOY-B	5.762951	2	0.056052
Sprg7& YOY-B	5.926763	2	0.051644
Spr10& YOY-B	5.830001	2	0.054204
YOY-A& YOY-B	4.421909	2	0.109596

==========

Table A5. Dispan output (*.dst) files with H, Gst andNei's Da distances (4 microes, 1 mtDNA)

==========

```
 Average heterozygousity and its standard error
(population 1) R128 1999:   0.558072   0.158328
(population 2) Fall 2007:   0.652052   0.171507
(population 3) Spring 2007: 0.652607   0.167893
(population 4) Spring 2010: 0.620132   0.124153
(population 5) YOY-A 2010:  0.639108   0.164569
(population 6) YOY-B 2010:  0.581109   0.158857

 All loci  Gst 0.190111 Ht 0.683821 Hs 0.553819
 Locus 1   Gst 0.040658 Ht 0.715856 Hs 0.686751
 Locus 2   Gst 0.925032 Ht 0.293275 Hs 0.021986
 Locus 3   Gst 0.177149 Ht 0.853952 Hs 0.702675
 Locus 4   Gst 0.125921 Ht 0.864696 Hs 0.755813
 Locus 5   Gst 0.129394 Ht 0.691324 Hs 0.601870

matrix: DA distances

              1         2         3         4         5
     2    0.3263
     3    0.2095    0.2301
     4    0.3000    0.3125    0.2756
     5    0.6142    0.5822    0.5245    0.5521
     6    0.2443    0.2556    0.2522    0.3115    0.4615
```

Fstat for windows, V2.9.3 2 - Microsatellite analysis results

Table A6. Allele frequencies in total material by locus, population and individual no.

****

	pop1	**pop2**	**pop3**	**pop4**	**pop5**	**pop6**	**All_W**	**All_UW**
Locus: LS19								
N	11	8	12	12	6	9		
p: 115	0.000	0.063	0.083	0.000	0.000	0.000	0.026	0.024
p: 121	0.000	0.063	0.000	0.000	0.000	0.000	0.009	0.010
p: 124	0.000	0.063	0.000	0.042	0.000	0.056	0.026	0.027
p: 127	0.045	0.000	0.000	0.042	0.000	0.000	0.017	0.015
p: 130	0.500	0.375	0.375	0.417	0.333	0.611	0.440	0.435
p: 133	0.182	0.063	0.167	0.250	0.333	0.167	0.190	0.193
p: 136	0.136	0.313	0.333	0.250	0.250	0.111	0.233	0.232
p: 139	0.136	0.063	0.000	0.000	0.000	0.056	0.043	0.042
p: 145	0.000	0.000	0.042	0.000	0.083	0.000	0.017	0.021
Locus: LS34								
N	9	2	1	7	3	4		
p: 144	0.000	0.000	0.000	0.071	1.000	0.000	0.135	0.179
p: 147	1.000	1.000	1.000	0.929	0.000	1.000	0.865	0.821
Locus: LS54								
N	8	1	5	6	6	7		
p: 161	0.000	0.000	0.000	0.000	0.333	0.000	0.061	0.056
p: 165	0.063	0.000	0.200	0.417	0.083	0.286	0.197	0.175
p: 169	0.000	0.000	0.100	0.000	0.250	0.143	0.091	0.082
p: 173	0.063	0.000	0.200	0.000	0.000	0.000	0.045	0.044
p: 177	0.063	0.000	0.100	0.000	0.000	0.071	0.045	0.039
p: 181	0.063	0.500	0.100	0.500	0.000	0.143	0.167	0.218
p: 185	0.063	0.500	0.100	0.000	0.167	0.286	0.136	0.186
p: 189	0.375	0.000	0.000	0.083	0.083	0.071	0.136	0.102
p: 193	0.313	0.000	0.200	0.000	0.083	0.000	0.121	0.099
Locus: AOX23								
N	6	5	10	6	7	9		
p: 92	0.000	0.000	0.000	0.000	0.143	0.000	0.023	0.024
p: 95	0.333	0.400	0.300	0.000	0.000	0.167	0.198	0.200
p: 98	0.000	0.300	0.200	0.333	0.571	0.056	0.233	0.243
p: 101	0.167	0.000	0.150	0.333	0.000	0.056	0.116	0.118
p: 110	0.000	0.000	0.000	0.083	0.000	0.000	0.012	0.014
p: 113	0.167	0.000	0.100	0.000	0.000	0.056	0.058	0.054
p: 116	0.083	0.000	0.000	0.167	0.000	0.000	0.035	0.042
p: 119	0.083	0.000	0.050	0.000	0.000	0.000	0.023	0.022
p: 122	0.000	0.100	0.000	0.000	0.143	0.278	0.093	0.087
p: 125	0.000	0.000	0.050	0.083	0.000	0.111	0.047	0.041
p: 128	0.167	0.000	0.000	0.000	0.071	0.167	0.070	0.067
p: 131	0.000	0.100	0.050	0.000	0.071	0.056	0.047	0.046
p: 134	0.000	0.100	0.000	0.000	0.000	0.000	0.012	0.017
p: 137	0.000	0.000	0.000	0.000	0.000	0.056	0.012	0.009
p: 140	0.000	0.000	0.050	0.000	0.000	0.000	0.012	0.008
p: 146	0.000	0.000	0.050	0.000	0.000	0.000	0.012	0.008

****

Gene diversity (heterozygosity) per locus and pop. 1-6

LS19	0.700	0.795	0.750	0.727	0.767	0.618
LS34	0.000	0.000	NA	0.143	0.000	0.000
LS54	0.804	NA	1.000	0.667	0.867	0.881
AOX23	0.933	0.850	0.900	0.867	0.714	0.903

****

number of alleles sampled by locus. pop. 1-6 & total

LS19	5	7	5	5	4	5	9
LS34	1	1	1	2	1	1	2
LS54	7	2	7	3	6	6	9
AOX23	6	5	9	5	5	9	16

****

Allelic Richness per locus and population 1-6

based on min. sample size of: 1 diploid individuals.

LS19	1.710	1.792	1.743	1.728	1.773	1.614	1.719
LS34	1.000	1.000	1.000	1.143	1.000	1.000	1.238
LS54	1.792	2.000	1.933	1.621	1.848	1.846	1.879
AOX23	1.864	1.800	1.868	1.803	1.670	1.889	1.879

****

F_{is} by locus and population 1-6 :

LS19	-0.299	0.056	0.222	-0.031	-0.087	0.101
LS34	NA	NA	NA	0.000	NA	NA
LS54	0.222	NA	0.600	0.750	0.231	0.514
AOX23	0.821	0.529	0.667	0.808	0.800	0.262
All	**0.302**	**0.301**	**0.516**	**0.490**	**0.300**	**0.313**

==========