Chapter 7

Evaluation of Genetic Diversity in Fish Using Molecular Markers

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Additional information is available at the end of the chapter

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1. Introduction

The variety of plants, animals and microorganisms on Earth, along with the tremendous diversity of genes in these species and the high variety of ecosystems on the globe are all constitutive parts of what is called "biodiversity". It is globally recognized that the conservation of nature and of its biological diversity should represent one of the priorities of the current human society. An important number of factors, sometimes interconnected, like climate change, pollution, anthropic activities destroying habitats for agriculture or logging, excessive exploitation of species with economic importance, hunting or poaching, represent serious treats for biodiversity.

The loss of biodiversity is a serious concern for multiple reasons, as a "healthy" biodiversity can provide various natural benefits including ecosystems services (contribution to climate stability, protection of water resources, nutrient storage and recycling, etc.), biological resources (food, medicinal and pharmaceutical resources, diversity in genes and species, breeding stocks and population reservoirs, etc.) and social benefits (research and education, recreation and tourism, etc.) [1].

Despite of well-known importance of biodiversity, human activity has been causing massive extinctions. In 2005, the report released by the Millennium Ecosystem Assessment pointed out a substantial and largely irreversible loss in the diversity of life on Earth, with some 10-50% of the well-studied higher taxonomic groups threatened with extinction, due to human actions. Over the past few hundred years, humans have increased species extinction rates by as much as 1000 times background rates that were typical over Earth's history [2]. Species are being lost at a rate that far exceeds the emergence of new species. The current extinction problem has been called the 'sixth extinction', as its magnitude compares with that of the other five mass extinctions revealed in geological records [3, 4].

The conservation of biodiversity at all levels from genes to ecosystems represents a global concern. Thus, the genetic diversity within species (between populations as well as among individuals within populations) as part of biodiversity is the result of the degree of variation at different levels (nucleotide, gene, chromosome, and genome). The presence of genetic variation plays an important role in species/populations survival and in their successful evolution in response to both short-term and long-term environmental changes [5]. To prevent the problems of genetic defects caused by inbreeding, species need a variety of genes to ensure successful survival. The decrease of this variety also translated as reduced genetic diversity is correlated with enhancing the chances of extinction.

The group of fish comprises over 32,000 species and an inestimable number of individuals inhabiting a large territory, from depths of 6000-7000 m on the bottom of oceans and seas to mountain waters found at an altitude of 2000 m. Thus, the fish group presents the most significant species variety among vertebrates [6]. The importance for humans derives mainly from economic significance, with aspects including consumption as food source, use in industry, aquaculture and fish farming. Overfishing and poaching along with habitat destruction (including water pollution, the building of dams, removal of water for use by humans, and the introduction of exotic species) represent the major threats to fish species and populations and currently, according to 2014 IUCN Red List, 2172 fish species are threatened with extinction [7].

Because of their life environment fish are more difficult to monitoring and study than terrestrial animal and to recover important information about fish populations is very challenging.

Sturgeons hold an important position in the category of the most threatened fish species in the world, mainly due to their particular scientific and commercial importance. Sturgeon species and the distantly related paddlefish as well as some extinct families are reunited in the Order Acipenseriformes and are generally regarded as "living fossils" and as the most primitive surviving bony fish. Sturgeons present some relict characters that differentiate them from other fish species and prove their ancient origin. The skeleton is primarily cartilaginous, with partial ossification only in cranium and maxilla, the posterior vertebrae continue far out into the dorsal lobe of the caudal fin (heterocercal condition), branchiostegal rays are absent or inconspicuous, undifferentiated vertebrae, ganoid scales only in caudal part of the body, five rows of bony scutes along the body. Sturgeons have a protrusible mouth, toothless in adults, a prominent snout with four barbells located about midway between the mouth and the snout tip [8].

Their extinct relatives (Infraclass Chondrostei) date to the Devonian (about 350 million years ago) and *Acipenser* fossils were found in the Western of North America dating from Upper Cretaceous. At present time within Acipenseriformes only two families exist, *Acipenseridae* and *Polyodontidae,* the first one counting 25 species inhabiting rivers, lakes, coastal waters and inner seas from the Northern hemisphere. The Acipenseriformes species are strictly Northern hemisphere fishes, some being resident to fresh water, while others are anadromous. Sturgeons are reunited in *Acipenseridae* family with four genera – *Acipenser*, *Huso*, *Scaphirhynchus* and *Pseudoscaphirhynchus*.

The area comprising the Black, Caspian and Aral Seas (Ponto-Caspian region) and the rivers that are tributaries to these seas, is the area with the greatest species diversity despite the fact the most of the populations in the region are facing extinction [9]. Currently in the Danube River three anadromous sturgeon species *Huso huso* (Beluga sturgeon), *Acipenser stellatus* (Stellate sturgeon) and *Acipenser gueldenstaedtii* (Russian sturgeon) and a potamodromous species *Acipenser ruthenus* (Sterlet sturgeon) are found [10]. The others two species that were present in the past in this area (*Acipenser sturio* – European sturgeon and *Acipenser nudiventris* – ship sturgeon) are considered to be extinct [11].

From ancient times, sturgeons had a great economic impact for the Danube region and they were an important part of the welfare of the local communities from this area. Due to human intervention, a decline of the sturgeon populations in this river has been observed starting with the 19th century, but much more accentuated in the 20th century. In Romania, in the first decade after the communist regime decline an overexploitation of sturgeon from Lower Danube has occurred mainly due to the absence of a legislation that protects these species [12, 13]. Thus, between 2002 and 2005 the sturgeon captures have severely diminished from 37 tons in 2002 to 11 tons in 2005. As a consequence of this evolution in 2006 the Romanian government adopted a law according to which the fishing of sturgeons for commercial purposes is prohibited for a period of 10 years. A solution to counter-balance the depletion of wild population was the one of sturgeon breeding in aquaculture. In the fish farms from Romania several species are preferred, especially Danube sturgeons as beluga sturgeon, stellate sturgeon and Russian sturgeon, considered to be ideal for obtaining caviar of superior quality and sterlet sturgeon that have the advantage of a very tasteful meat. Beside these species, the Siberian sturgeon (*Acipenser baerii*) and different inter-specific hybrids are also raised in aquaculture.

The causes for the dramatic decline of sturgeon populations all over the world are related mostly to the changes that are taking place in their habitats: pollution, dams and other constructions that are blocking the migration into the river for reproductive purposes, overfishing and poaching, mainly to provide caviar for the black market. Another factor that has a negative impact on the sturgeon populations is the practice of stocking the rivers with individuals from aquaculture, which becomes more and more popular with the years. Uncontrolled restocking presents as consequence the decrease of genetic diversity within and between populations, mainly due to the reduced number of adult individuals from the wild used as genitors for reproduction in aquaculture. Also, other practice with disastrous consequences for the populations is the one of restocking with inter-specific hybrids incorrectly labeled as individuals of native species. The inter-specific hybridization phenomenon is relatively frequent in sturgeons both in the wild and in aquaculture. Such hybrids were caught in the Danube River [14, 15] or are produced and raised in sturgeon farms. The species identifications based only on morphology might be misleading sometimes and molecular methods were proposed for species identification and caviar labeling [15-18].

Other fish species with a high ecological and economic importance are represented by salmonids. Romania is one of the European countries characterized by the presence of a significant number of wild salmonids populations and by an important potential for the development of intensive breeding of salmonids in aquaculture. The Salmoniformes order represents a heterogeneous group of fish reunited in one family *Salmonidae*, with three subfamilies (*Coregoninae* - whitefish and ciscos; *Thymallinae* – graylings; *Salmoninae* – trout, salmon and charr), nine genera and roughly 68 species [19]. The largest of salmoniform fishes are considered to be Chinook salmon (*Oncorhynchus tshawytscha*) and Danube huchen (*Hucho hucho*), that might reach at 1.5 m in length and a weight around 60 kg. Salmonids are characterized by body and fins that are streamlined and symmetrical, being covered with small and cycloid scales. All fins have soft rays. The representatives of this order possess several primitive anatomical features that are characteristics for an early stage in the evolution of modern bony fishes, like a small, fleshy adipose fin located between the dorsal fin and the powerful caudal fin [20].

They are native to the cooler climates of the Northern Hemisphere, but have been widely introduced around the world for angling and aquaculture.

In the Romanian fauna the following salmonid species are present: *Salmo trutta fario* (brown trout), *Salmo labrax* (Black Sea salmon), *Salvelinus fontinalis* (brook trout), *Hucho hucho* (huchen or Danube salmon), and *Thymallus thymallus* (grayling). The European whitefish (*Coregonus lavaretus maraenoides*) was introduced starting with 1957 in Lake Rosu, Bicaz and Tarcau rivers, the biological material originating from Poland and Russia. Unfortunately, there are no other studies regarding the adaptation of this species in the water systems where has been introduced. Apart the salmonids from the wild fauna previous mentioned the following species are bred for commercial purposes in aquaculture: brown trout, brook trout and rainbow trout (*Oncorhynchus mykiss*) and a series of hybrids of natural and fishery species.

The taxonomy of *Salmo* is still a matter of controversy [21] since more than 60 synonyms for varieties of brown trout and more than 20 for varieties of Atlantic salmon were described. The number of *Salmo* species recognized varies considerably not only because of highly phenotypic variation (body shape, colour, etc.), but also because the species inhabit and are adapted to very different habitats over large distribution areas. Due to taxonomic ambiguities, authors often refer to brown trout as *Salmo trutta* species complex [22, 23].

Salmo trutta comprises several distinct ecological and geographical morphs and with respect to this is still controversy as far as their classification as species or subspecies is concerned [24, 25]. Based on morphological and ecological variations, the existing populations of *Salmo trutta* from distinct areas are grouped into different taxa: i) Black Sea populations – *Salmo labrax*, ii) Caspian Sea populations – *Salmo caspius*, iii) Aral Sea populations – *Salmo oxianus* and iv) Mediterranean Sea populations – *Salmo macrostigma* [26].

The brown (common) trout (*Salmo trutta* morpha *fario* and *Salmo trutta* morpha *lacustris*) and the sea trout (*Salmo trutta* morpha *trutta*) are fish of the same species, considered by some taxonomists different subspecies in order to distinguish the anadromous *Salmo trutta trutta*, living in the sea and migrating in freshwater only to spawn, from *Salmo trutta fario*, residing in freshwater and the lake dwelling form *Salmo trutta lacustris*. Instead, other authors consider that these do not necessarily represent monophyletic groups [27].

In Romania, *Salmo trutta fario* (Linnaeus, 1758) is widely spread in a large number of water streams from the mountain area, along the Carpathian Arch, whereas the Black Sea trout, *Salmo* *labrax* (Pallas, 1814) is endemic to the Black Sea area and migrates for reproduction in the Danube River and its tributaries. Nowadays only few individuals are captured annually along the Black Sea coast and sporadically in the Lower Danube River. Due to the present situation of the species several measures were adopted to protect it. Thus, the fishing is completely prohibited and the Black Sea trout is included on the Red List of Danube Delta Biosphere Reserve [8].

Salvelinus fontinalis (Mitchill, 1815) is predominantly raised in fish farms for food consumption, but a low number of wild populations are still present in the Romanian mountain waters.

Thymallus thymallus (Linnaeus, 1758) is the only native salmonid species for which no imports of biological material and restocking programs were completed in Romania [26].

Until two or three decades ago, excellent habitat conditions for Danube huchen (*Hucho hucho*, Linnaeus, 1758) still existed in many rivers in Romania. The historical range of this species included the majority of the Carpathian river systems [28], but hydropower development, river pollution, and overfishing and poaching led to drastic declines in the area inhabited by huchen [29], and the species is now extinct in the Mures, Timis, Cerna, Olt, Arges, and Ialomita river systems. Data on the current area of huchen occurrence in Romania are fragmentary, and the species only occurs in a few rivers, including, among others, the Tisa River and its tributaries (Viseu, Ruscova, Crasna, Bistra, Vaser, Somes, and Cris) and in the Siret River.

In relation to the main rivers from Romania, there are 10 hydrographic basins in which significant salmonid populations are found. Nowadays is a well-known fact that the most prevalent salmonid species is the brown trout, while the most threatened are the Danube huchen and the Black Sea salmon.

Both sturgeons and salmonids are of crucial importance for Romanian fauna, having also a significant socio-economic value. In this context, well documented studies regarding the biology, taxonomic classification and ecology of the species were performed [10, 28, 30-32]. The development of technologies based on DNA markers has had a tremendous impact on animal genetics in generally, and changed the way in which studies were conducted inclusive in population genetics, phylogeny, phylogeography and conservation. Thus, in a progressive way, studies aiming at analyzing molecular aspects in sturgeons and salmonids populations from Romania founded their place in the research field, although the number of such studies is still relatively low.

2. Molecular markers for population genetics and conservation

The definition of "marker" in wide sense is "something that serves to identify, predict, or characterize" [33]. In biology, the markers refer to any stable variation, which is heritable and can be measured or detected by an appropriate method. Such variations are produced at different levels for example, morphological, gene, chromosomal, biochemical or genomic. The markers that represent variations that appear at the DNA level are so-called *molecular mark-*

ers. The molecular markers occupy specific places in the genome and possess the role to "mark" the position of a specific gene or the inheritance of a particular character.

All organisms are subject to mutations that appear when genetic material fails to copy accurately or as a consequence of interactions with the environment, leading to genetic variation or polymorphism. Individuals of a species are distributed into more or less separate groups called populations, distributed over the species range. The genetic variation of a species is distributed both within and between populations. The diversity at the gene level is often referred to as intra-specific variability as it represents biological diversity within a single species. The researchers are very interested in assessing genetic variation within and between populations and detecting similarities as well as differences between individuals/ populations in order to establish optimum conservation strategies.

For this variation to be useful in different genetic studies it is necessary to be inherited and to be perceptible for the researchers, whether is recognizable as phenotypic variation or as a genetic mutation detectable by different molecular techniques. At the DNA level the genetic variation is represented by point mutations called also single nucleotide polymorphisms (SNPs), indels (insertions or deletions of nucleotide sequences), inversion of a segment of DNA within a locus, and rearrangement of DNA segments around a locus of interest. As the mutation rate is very low the evolution through mutation is extremely slow. The process of mutation is the only way in which genetic variation is created and in the lack of mutations there would be no biological diversity.

DNA marker technology can be applied to reveal these mutations. Large deletions and insertions determine shifts in the size of DNA fragments resulted consequently digestion by restriction enzymes, and are among the easiest type of mutations to detect by electrophoresis on agarose gel; smaller indels require DNA sequencing or more elaborate electrophoretic techniques, while the SNPs can be easily detected by DNA sequencing [34].

By using molecular markers is possible to observe and exploit genetic variation across the entire genome. Thus, the application of molecular markers in fish allowed recording rapid progress regarding the study of genetic variability and inbreeding, parentage determination, species identification, genetic linkage map construction for aquaculture species, identifying **Quantitative Trait Loci (QTL)** related to specific traits for marker assisted selection. One of the classifications of the molecular markers refers to **markers of type I** as markers associated with genes having a known function and **markers of type II** as markers associated with unknown genomic segments [35]. The type I of DNA markers were not initially consider to be appropriate for genetic studies in fishes, but over time it became clear that these markers are very important both for the study of wild populations and aquaculture. Thus, this of markers has become very important for studying the phenomenon of linkage and for QTL mapping, being of great use in comparative genomic studies and for identification of candidate genes for quantitative traits in different fish species raised in aquaculture. An important number of studies were focused on the elucidation of the molecular basis of economically important traits in different species of salmonids. For example in the case of Atlantic salmon the mapping the QTLs was performed for the loci correlated with viral disease resistance [36], flesh color and growth traits [37], salinity tolerance [38], late sexual maturation [39], etc.

The type II of molecular markers (**RAPD, AFLP, microsatellites**) is considered to be non-coding. Such markers are used in population genetic studies aiming to characterize the genetic diversity. The type II of DNA markers proved useful in identifying species, populations and subpopulation, but also in identifying interspecific hybrids.

A second classification of markers is the one regarding their position in the cell. Thus we can distinguish between **nuclear and mitochondrial DNA (mtDNA) markers**, depending on their localization in the nuclear or mitochondrion genome.

The molecular markers might be highlighted by a variety of techniques that differ by difficulty, repeatability, cost and nature of polymorphism that is detected. The **DNA markers** detected by Polymerase Chain Reaction (PCR) are the most frequent used in assessing the genetic diversity and have a particular role in conservation. By PCR is possible to amplify DNA sequences up to several million times, so these markers present the advantage of nonlethal sampling. Small amount of tissue (fin clips or scales) are sufficient for analysis and is not necessary that the individuals to be sacrificed for sampling. This can be an important feature when evaluating genetic change in protected or declining populations and for providing access to DNA of ancient or archived tissue samples. It can provide information about genetic diversity over extensive temporal and spatial scales, especially for populations that no longer exist. Taken together, these characteristics suggest capability to monitoring populations that are small, exploited or declining.

Depending on the primers used for PCR amplification these markers can be divided in two groups: **(i) PCR markers for target sequences** – in this case the fragment of interest is amplified with two specific primers and **(ii) PCR markers for arbitrary sequences** – one primer with a arbitrary nucleotide sequence is used; the primer binds to randomly in the genome resulting unknown DNA fragments. The primer used in this type of techniques is usually short (of 8-10 nucleotides) and so the probability for it to bind at multiple sites in genome increase.

In the first category are found molecular markers like PCR-RFLP (PCR Restriction Fragment Length Polymorphism), PASA (PCR Amplification of Specific Alleles), SNP (Single Nucleotide Polymorphism), repetitive DNA sequences (minisatellites, microsatellites, etc.) [34]. The second category includes markers like RAPD (Randomly Amplified Polymorphic DNA), AP-PCR (Arbitrary Primed-PCR) and AFLP (Amplified Fragment Length Polymorphism), but these are less preferred comparing with PCR markers for target sequences due to the difficulty of analysis and lack of results accuracy and reproducibility.

PCR-RFLP

The analysis of these markers involves the amplification by PCR of a specific DNA region comprising one or more polymorphic sites for restriction enzymes (RE). With the increasing number of so-called "universal" primers available, can be targeted DNA regions that are relatively conserved among species. In addition, PCR products can be digested with restriction enzymes and visualized by ethidium bromide staining due to the increased amount of the resulting DNA amplification reaction. The ability of RFLP markers to highlight the genetic variation is relatively low compared with the one of other markers. Substitutions, insertions, deletions and rearrangements of the regions containing restriction sites are probably quite widespread in the genomes of many species, but the probability that they exist at the locus that we want to study is quite low. Because the difference in size between the restriction fragments is usually large these can be easily separated by agarose electrophoresis. The disadvantage of PCR-RFLP is that presents a relatively low level of polymorphism and requires knowing the sequence of the fragment amplified by PCR, fact that makes difficult to establish new markers.

In genetic studies regarding sturgeons the PCR-RFLP markers were mainly used in two directions: to assess the genetic diversity of sturgeon populations [40, 41] and for species identification and implicit for caviar traceability [42, 43]. Wolf et al. [42] has identified species specific restriction profiles in a fragment from the mitochondrial gene *cytochrome b* (*cyt b*) in 10 species from *Acipenser* and *Huso* genera. A similar study was performed by Ludwig et al [44] for 22 sturgeon species, an accurate identification being possible by analyzing the restriction profiles resulted consequently the action of five REs on a fragment of 1121 bp from *cyt b* gene. Panagiotopoulou et al. [43] proposed a molecular method based on PCR-RFLP to distinguish between Atlantic (*Acipenser oxyrinchus*) and European (*A. sturio*) sturgeon. The discrimination between the two species is difficult to be done exclusively by morphological traits, especially in their early life stages, while the application of two REs allowed the clear and unambiguous discrimination of 132 specimens of Atlantic and European sturgeon.

The identification of acipenserid species by PCR-RFLP presents the difficulty of correct diagnostic for species that are closely related from genetic and evolutionary point of view, like is the case of *A. gueldenstaedtii* / *A. persicus* or *Scaphirhynchus* genus species. Moreover, the analysis of length polymorphisms for mitochondrial DNA fragments put in evidence only the genetic variability originating from the maternal genitor, as the mtDNA is almost exclusively maternally inherited. This fact might lead to misinterpretations in case of inter-specific hybrids diagnostic and/ or an ancient introgression, both phenomena occurring in sturgeons [15, 45].

PCR-RFLP markers were used also in salmonid species for the genetic diversity and phylogeography analyses in different populations of brown trout [46, 47], charrs [48], grayling [49], etc.

Microsatellites also referred to as “simple sequence repeat” (SSR) [50] represent short repetitive sequences of 2-9 bp, wide spread in the genome and with a significant level of polymorphism. These markers are numerous in vertebrates, in fish appearing in every 10kb [51]. The majority of microsatellite loci are relatively small sizes, being amplified easily by PCR. Up to 70% of loci present dinucleotide repeats, the (AC) motif being the most common in the vertebrate genome [52]. The main features of microsatellites are co-dominant inheritance, high degree of polymorphism, hypervariability, higher mutation rate than standard. Through microsatellites analysis is possible to infer the genetic profile of an individual (genetic fingerprint) and to establish the relationships between individuals. Some microsatellites have a high number of alleles for a locus within population and are very suitable to identify the genitors and their progeny in hybrid populations. The higher level of allelic variation at microsatellite markers make them useful for addressing questions related to genetic structure, particularly where genetic differentiation may be limited. On the contrary other microsatellites

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present a low number of alleles or even a fixed allele and are more appropriate for phylogeny or species identification. The utility of microsatellite markers can be determined depending on the polymorphic information content (PIC), defined as the capacity of the marker to detect a polymorphism in the population [53]. PIC is direct correlated with the number of detected alleles and their frequency, a higher number of alleles determining a higher value for PIC. By comparing the values for PIC in different markers is possible to obtain valuable information about their power and efficiency in population genetic and conservation studies.

The primers designed for microsatellites amplification in a species give also cross-amplification for similar loci in related species, this being an important benefit in analyzing populations that are small or at the brink of extinction [54]. Microsatellite markers are widely used for population genetic and conservation studies in fishes.

The first studies based on microsatellite loci analysis were initiated at the North-American species, where it aimed at isolation and description of disomic loci [55, 56, 57].

The identification and characterization of new loci is complicated by the polyploidy of the sturgeon species. Lots of potential useful microsatellites were eliminated from analysis since these were polysomic and thus, they complicated the interpretation of the inheritance mode and of genetic variation within and between populations. Once a disomic set of microsatellites is established, this fact permits to analyze the genetic diversity and structure of wild populations and aquaculture stocks. Such disomic loci were isolated and characterized also for *A. naccarii* [58, 59] and *A. persicus* [60]. In the case of Ponto-Caspian sturgeons, a few studies based on microsatellites analysis in *A. stellatus* from the Caspian Sea [61] and, respectively, in *A. gueldenstaedtii* from the North-West of the Black Sea, North of the Caspian Sea and Azov Sea [62] were performed.

Beside their high applicability in for inferring the genetic diversity and structure of the wild populations, the microsatellites are appropriate for aquaculture stocks evaluation, selection of breeders and proper conservation. Different studies were conducted in several sturgeon species (*A. transmontanus, A. fulvescens, A. naccarii, A. sturio,* etc.) for *ex-situ* conservation purposes. The obtained data were used in the management program to adopt appropriate conservation methods.

In salmonids, the microsatellites were successfully applied in phylogeography studies [22], in determination of genetic variation in wild and farmed fish populations [63], in inferring the genetic diversity within population, fine-scale genetic differentiation and relationship of populations [64], in assessment of stocking impact on wild populations [65].

The mitochondrial genome possesses certain characteristics (compact organization, maternal inheritance, hundreds to thousands of copies per cell, rapidly evolving, reduced recombination rate, and higher mutation rates compared to those of nuclear genes) that make it useful in population genetics and phylogeny studies. mtDNA analysis in sturgeons is a suitable method for the characterization of species and populations, providing useful information for the management of conservation activities. For intraspecific studies, the most commonly used marker is the mitochondrial control region (D-loop) due to the relatively high degree of nucleotide variation. Studies to discriminate species/populations/aquaculture strains based on the analysis of D-loop region polymorphisms were performed for several species of sturgeon, such as *A. gueldenstaedtii* [66], *A. sinensis* [67], *A. stellatus* and *A. baerii* [68].

The gene coding for *cyt b* is a useful marker for identifying different species of sturgeon based on specific polymorphisms found at this level. A 648 bp fragment of the gene coding for *cytochrome oxidase subunit I* (*CO I)* is considered a real "barcode" for vertebrates and is useful for species identification by *DNA barcoding* technique.

Genes with highly conserved sequence and slow evolution like mitochondrial ribosomal genes (16SrRNA and 12SrRNA genes) are preferred when is about inferring the phylogenetic relationships of fishes at different taxonomic levels. 12SrRNA gene is considered a promising tool for tracing the history of more recent evolutionary events and it has been widely used to study the phylogenetic relationships among different levels of taxa such as families, genera and species [26].

In salmonid species mtDNA has proven to be useful for phylogeographical studies [69] and for analysis of spatial and temporal population structure [70]. Based on the analysis of the mitochondrial D-loop marker Bernatchez [22] showed that there are five different main lineages of *Salmo trutta* in Europe: Atlantic, Danubian, Marmoratus, Mediterranean and Adriatic. Analytical techniques for mtDNA include indirect methods such as the analysis of RFLP markers or the direct analysis of mtDNA sequences.

3. Factors influencing the genetic diversity of populations

The genes are transferred from one generation to the next, and every individual has two copies of each gene, one which is inherited from the maternal genitor, the other from the paternal genitor. The DNA sequence of a specific locus, either a gene or a non-coding marker, may present some differences, resulting different variants of the same locus. Such variants of a specific locus are called alleles and their existence implies the genetic variation existence.

i. **Mutation** is in wide sense the process of random change of the DNA sequence and represents a process by which new alleles are created. As the mutation rates at nuclear and mitochondrial genome are very slow, the evolution through mutation is so slow that it is generally impossible to detect it from one generation to another. However, mutation is important as a source of genetic variation. The process of mutation is the only way in which genetic variability is created, and without mutations there would be no biological diversity.

ii. **Gene flow.** A population acquires new alleles mostly through the immigration of individuals from surrounding populations (gene flow) and through mutations. Apart from these two processes the number of alleles in a population is determined by the size of the population. Gene flow is a change in allele frequency that occurs due to migration of individuals among populations. It is possible that individuals that are moving into a new population to bring new alleles which are not present in that population or that they are in frequencies that differ from the allele frequencies of that population. So the gene flow increases the genetic variation within a population, but tends to make populations genetically similar to each other. If migration between populations occur in large numbers of individuals and the level of gene flow is significantly high, the populations will have the same alleles in the same frequencies and it will be one single population.

iii. **Genetic drift** represents a random modification in allele frequency of population that occurs if a population size is not infinite. In populations that are of limited size, allele frequencies will change randomly from one generation to the next. In the short term, over a few generations, a result of genetic drift would be the increasing or decreasing of allele frequencies in a random, unpredictable way. In the longer term, the main result of genetic drift is loss of genetic variation as by chance some of the alleles that exist in the parent generation may not be passed on to their offspring. The effects of genetic drift are strongest in small populations because the more impressive is the fluctuation of allele frequencies, and the sooner the loss of genetic variation. Genetic drift also results in different populations becoming genetically different from each other because different alleles will become more frequent or fixed in different populations.

iv. **Natural selection** is the gradual process by which biological traits become either more or less common in a population as a function of the effect of inherited traits on the differential reproductive success of organisms interacting with their environment. Natural selection appears because different genotypes have different fitness. Individuals with higher fitness survive and reproduce more than other individuals, so these genotypes become increasingly more and more frequent in populations. In different populations, parents of different genotypes pass their genes unequally to the next generation, leading to the genetic differences among isolated populations. So, genetic drift tends to make different populations genetically distinct from each other by chance, whereas natural selection tends to form genetically different populations due to environmental constraints. Consequently, the traits that have high fitness in one population, and evolve through natural selection, will be different from the traits that have high fitness and evolve through natural selection in another population. In generally, the natural selection, genetic drift and gene flow have an effect on genetic variation within populations and between populations. While the genetic drift and selection tend to reduce the variation within populations and increase the differences between populations, the gene flow increases the variation within populations, but makes populations similar.

v. **Inbreeding.** The birth of offspring resulted from reproduction between close relatives that occurs mainly in small and isolated populations. The consequence of inbreeding is the reduced viability and reproduction, as well as increased occurrences of diseases and defects, so called inbreeding depression [71].

4. Molecular studies for species identifications and genetic diversity assessing in sturgeons from Romania

The markers analyzed in sturgeon species and populations from Romania were nuclear markers (microsatellites) and mtDNA. The methodologies applied in the studies included microsatellites genotyping, PCR-RFLP and sequencing of mitochondrial markers. Thus, the DNA was isolated from biological samples consisting in small pieces of fin sampled without harming the animals (aspect very important in vulnerable populations) by a classic protocol with phenol-chloroform-isoamylic alcohol.

For mtDNA analysis the primers were designed based on DNA sequences for the interest markers retrieved from GenBank data base. The primers for microsatellites amplification were described in literature in different species and the cross-amplification for the similar species from Romania was tested.

The molecular markers were amplified by PCR or multiplex PCR in specific conditions established consequently several steps of reaction optimization. The microsatellites were analyzed by capillary electrophoresis in ABI Prism 310 Genetic Analyzer (Applied Biosystems) and the mitochondrial markers were sequenced by Sanger method, *dye terminator* variant, in ABI Prism 3130 Genetic Analyzer (Applied Biosystems). The raw data were processed and edited with dedicated computer programs. The interpretation of genotypic data was done by using specialized computer software for population genetics and phylogeny.

In the context of severe decline of sturgeon population from the Lower Danube several studies based on molecular marker analysis were performed in the recent years in Romania. In consequence, the studies were directed to assessing the genetic diversity in Lower Danube sturgeon populations based on microsatellites and to evaluate genetic variability in aquaculture strains in order to sustain the efforts of conservation.

The analyses regarding the genetic diversity were preceded by the correct species identification based on molecular markers for each individual. As mentioned before, the accurate detection of sturgeon species encountered in the Danube River can be very difficult, due to the plasticity of various external morphological features. The number of hybrid individuals occurring in natural conditions is unknown. However, the number of hybrids in natural waters could increase due to escapes from commercial farms where exotic species or genotypes and interspecific hybrids are regularly used for production purposes. So, decisions to initiate restocking programs for sturgeon conservation need to take into account, besides the socio-economical aspects and the assessment of genetic diversity, the correct diagnostic of fishes included in this type of programs.

For sturgeon species from the Lower Danube diagnostic a molecular methods was set-up by Dudu et al [15] based on the analysis of nuclear markers. In this study 84 individuals belonging to all four species from the Lower Danube (A. *stellatus, A. gueldenstaedtii, A. ruthenus* and *H. huso*) were analyzed. Initially 25 microsatellites were tested from which eight loci (LS19, LS34, LS39, LS54, Aox27, AoxD234, AnacE4, and AnacC11) that have demonstrated a good amplification, results repeatability and interspecific polymorphism in all of the four investigated species were selected. The genotypic data processing involves three successive statistical analyses including Factorial Correspondence Analysis (FCA), STRUCTURE assignation and NewHybrids status determination.

In a first step, the genotypes data were run in a FCA test, using GENETIX software only for the individuals considered being pure species based on morphology analysis. The FCA highlighted the differences between the four analyzed species. Four main clusters, each corresponding to one of the sturgeon species analyzed, were identified (Figure 1).

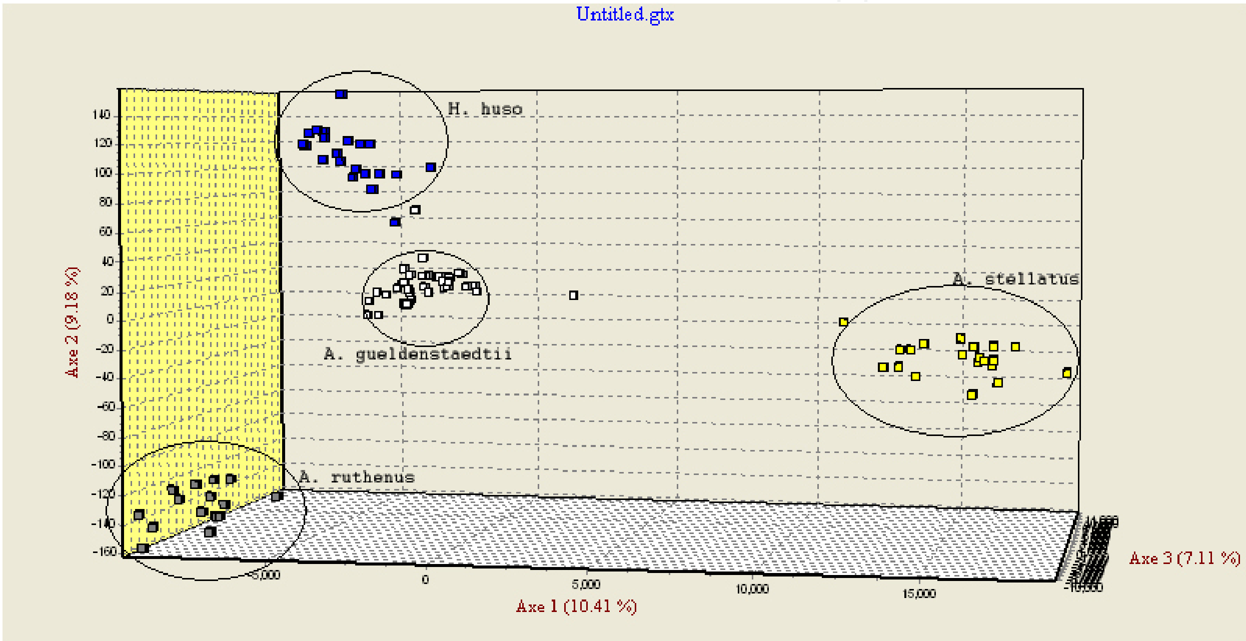

Figure 1. Factorial Correspondence Analysis (FCA) based on 8 microsatellite loci in the four pure species of Danube sturgeons [15].

In the second step, the genotypes data of putative hybrids, diagnosed such as based on morphometric indices were included in FCA analysis as supplementary individuals. A fifth category was highlighted including the putative hybrids, but also individuals that were considered as pure species, based on their morphology. The analysis had showed that only three of the four species hybridize each other, the putative hybrids displaying an intermediate position between *A. stellatus*, *H. huso* and *A. gueldenstaedtii.* Consequently, we deduced that *A. ruthenus* individuals should be eliminated from further tests (Figure 2).

In consequence in the third step, FCA was performed only on three pure species and hybrid individuals included as supplementary elements in analysis. The analysis grouped the pure species and the hybrids in distinct clusters. The hybrids and some of the individuals labeled as pure were occupying an intermediate position between the pure species groups, some of these being placed approximately in the middle of the triangle delimited by the three pure species, while others appear to be closer to *H. huso* and *A. gueldenstaedtii* (Figure 3).

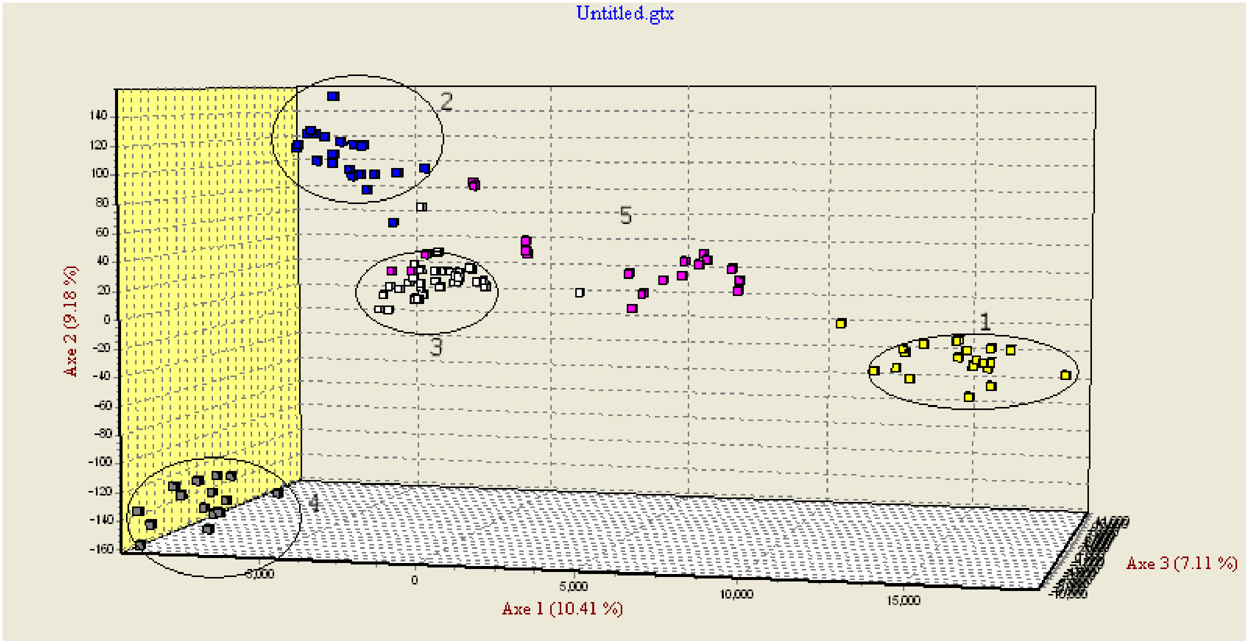

Figure 2. Factorial Correspondence Analysis (FCA) based on 8 microsatellite loci in the four pure species and hybrids of Danube sturgeons. (1) *A. stellatus*; (2) *H. huso*; (3) *A. gueldenstaedtii*; (4) *A. ruthenus*; (5) Hybrids and some pure individuals to be tested with other methods [15].

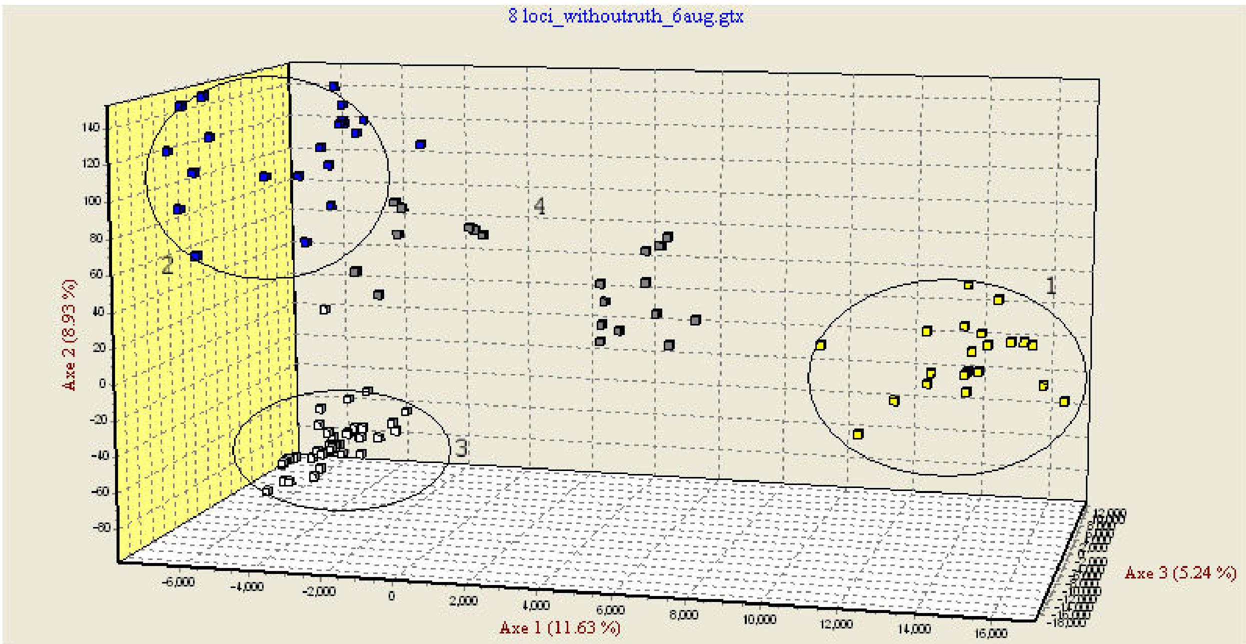

Figure 3. Factorial Correspondence Analysis (FCA) based on eight microsatellite loci in three pure species and hybrids of Danube sturgeons: (1) *A. stellatus*; (2) *H. huso*; (3) *A. gueldenstaedtii*; (4) Hybrids and some pure individuals [15].

The second statistical analysis of the genotype data was the assignment test with STRUCTURE, which confirmed the presence of five specific clusters: (1) *A. stellatus* pure species; (2) *H. huso* pure species; (3) *A. gueldenstaedtii* pure species; (4) *A. ruthenus* pure species; (5) hybrids (Figure 4). The individuals classified as pure based on morphology (with three exceptions) were strongly assigned in their corresponding species. The perfect correspondence between morphological and molecular/assignation determinations gives a great reliability to the results.

The hybrids confirmed by the FCA and STRUCTURE assignment test were analyzed together with their two genitor species using the NewHybrids software in order to distinguish between F1 and later hybridization steps.

By the three successive statistical analyses it is possible the diagnostic of surgeon individuals as pure species or hybrids, the method proposed showing a high efficiency in discriminating pure species specimens from F1, F2 and two kinds of backcross.

The molecular analysis for species detection is necessary for all sturgeon individuals captured in the Lower Danube River and implied in restocking programs and from aquaculture. A database with genotypic data can be created as the accuracy of the proposed method increases along with the number of reference individuals analyzed. Also, such database might be of a real use for designing efficient management plans for sturgeon populations.

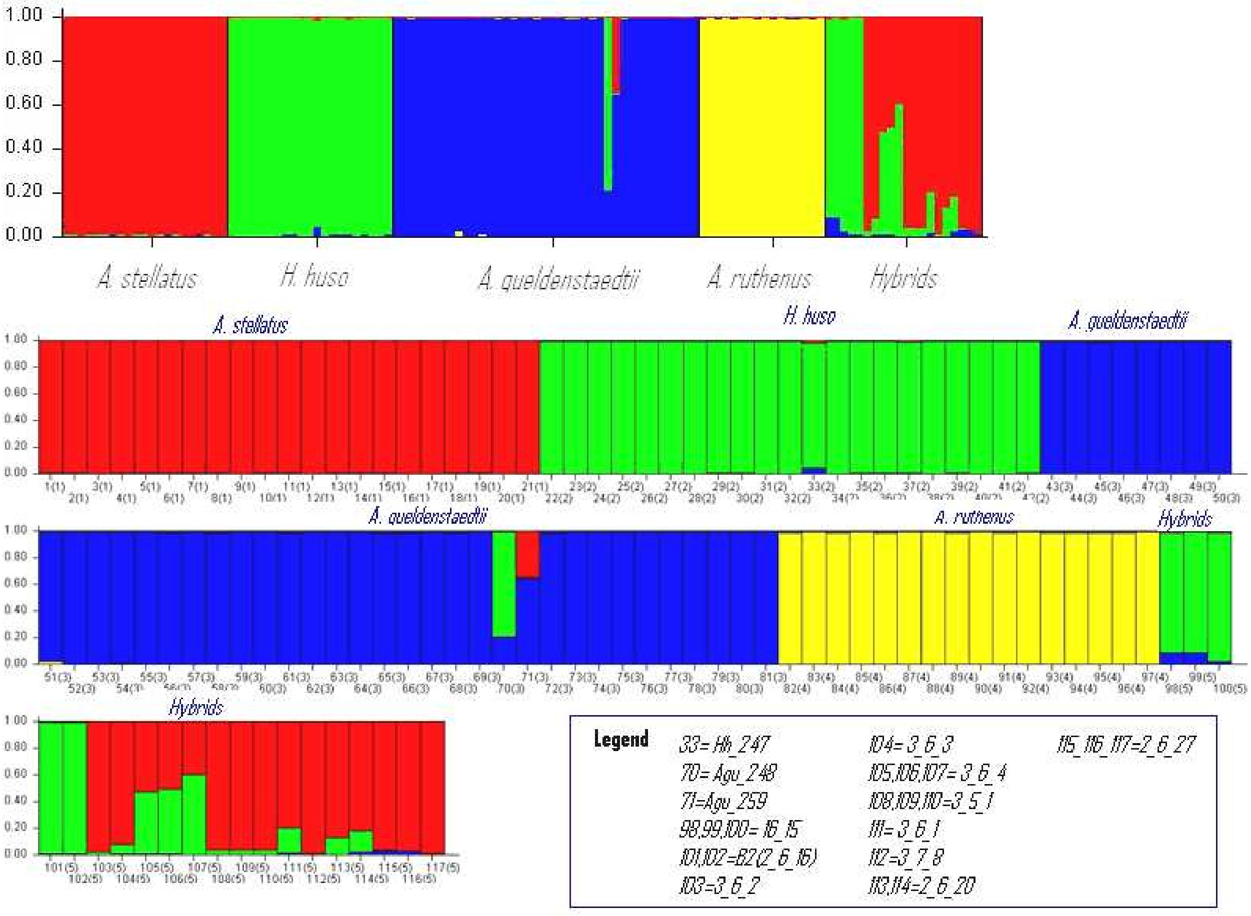

Figure 4. Assignation of the 84 sturgeons by STRUCTURE analysis based on eight microsatellite loci in four sturgeon species from Danube. Histograms represent the estimated membership coefficients (Q). Composite bars are expected hybrids [15].

When a hybrid is detected the origin of maternal species can be identified by analyzing mitochondrial markers. For example the analysis by sequencing or PCR-RFLP of *cytb* gene permits the identification of the maternal genitor involved in hybrid formation as is well known that the mitochondrion genome is exclusively maternally inherited. The species identification based on the analysis of mtDNA by different methods should be handled with precaution when we deal with species that can easily hybridize, like sturgeons. The mitochondrial marker analysis is suitable for pure species, but totally inefficient for hybrids.

The analysis of genetic diversity in the populations from the Lower Danube by microsatellites was hindered by the complexity of the genome in this group of fish.

All Acipenseriformes are divided into three separate groups depending on the number of chromosomes: (1) species with karyotypes comprising about 120 chromosomes; (2) species with 240 to 270 chromosomes; they are conventionally referred to as 250-chromosomes species; (3) species with around 370 chromosomes [72]. Two scales of Acipenseriformes ploidy have been proposed: (1) the "evolutionary scale": diploid species (extinct), tetraploid species (120-chromosomes), octoploid (250 chromosomes), and 12-ploid (370-chromosomes) species [73]; and (2) the "contemporary scale": diploid (120-chromosomes), tetraploid (250-chromosomes), and hexaploid (370-chromosomes) species [74].

Three of the four sturgeon species from the Lower Danube – *A. stellatus, A. ruthenus* and *H. huso* are considered to be functional diploid as the process of functional reduction of the genome is consider being almost completed in these species, while *A. gueldenstaedtii* is functional tetraploid, with an octaploid ancestor from which some loci are maintained. The analysis of a set of 12 microsatellites loci (AciG93, AciG198, AnacC11, AnacE4, Aox27, AoxD234, As002, LS19, LS34, LS39, LS54, Spl106) in 51 individuals of *A. gueldenstaedtii* from Lower Danube revealed that only two loci were disomic, while the others were polysomic with a number of 3-8 alleles per locus in an individual (Table 1).

Locus	Allele size (bp)	Maximum number of alleles per individual
AciG93	383-399	4
AciG198	184 - 188	2
AnacC11	144-204	4
AnacE4	320-356	3
Aox27	110-142	4
AoxD234	188-268	4
As002	92-140	8
LS19	115-154	4
LS34	121-151	4
LS39	85-157	4
LS54	117-237	4
Spl106	234-246	2

Table 1. Characteristics of 12 microsatellite loci in *A. gueldenstaedtii* from Lower Danube.

The analysis of genetic diversity by using microsatellite with polysomic pattern is facing the problem of correct determination of the genotype. For example, for a locus with tetrasomic pattern for which only three peaks are detected by capillary electrophoresis is hard to determine which allele has two copies. The method of *gene dosage* proposed by Jenneckens et al [75] by which the genotype can be determined by calculating the report of peak areas has proven to be inefficient in our case.

For the *A. stellatus*, *A. ruthenus* and *H. huso* population from the Lower Danube genetic diversity studies were performed based on microsatellite analysis. From an extended set of microsatellites isolated originally in the North–American sturgeon species *A. fulvescens* and *A. oxyrhinchus* and in Adriatic sturgeon – *A. naccarii*, seven loci (LS19, LS34, LS39, LS54, AnacE4, AnacC11, and AoxD234) that showed good results of amplification and a disomic pattern in all the three species of sturgeon from Lower Danube, were selected for the assessment of genetic diversity.

The analysis was performed in a total number of 158 sturgeon individuals (62 of *A. stellatus*, 54 of *H. huso* and 42 of *A. ruthenus*) that were captured in the river between 2001-2008 as part of a national scientific research study for restocking and monitoring.

The estimation of the genetic diversity was realized by inferring several statistic indices with Genetix software. Thus the average values of expected heterozygosity (H_E), observed heterozygosity (H_O) and the mean number of alleles/locus (MNA) were calculated.

The heterozygosity is the percentage of heterozygous loci in a population. The average values of H_O are similar for the three sturgeon population from the Lower Danube (0.431 – *A. stellatus*; 0.476 – *H. huso*; 0.4017 – *A. ruthenus*), while the average values of H_E are situated between 0.6409 (*H. huso*) şi 0.5634 (*A. ruthenus*). The highest values of H_O (0.4760) and MNA per locus (6.2875) were obtained for *H. huso*, which appears to have the highest variability among the analyzed populations.

Locus	Population								
	A. stellatus			*H. huso*			*A. ruthenus*		
	H_E	H_O	MNA	H_E	H_O	MNA	H_E	H_O	MNA
LS19	0.7053	0.5484		0.8033	0.6667		0.4963	0.4524	
LS34	0.1152	0.0862		0.0000	0.0000		0.3501	0.0976	
LS39	0.0000	0.0000		0.5492	0.3889		0.4963	0.4048	
LS54	0.8321	0.7119		0.7758	0.4800		0.4887	0.0476	
AnacE4	0.8100	0.5484		0.6540	0.3889		0.5635	0.1429	
AnacC11	0.7243	0.7833		0.6372	0.4259		0.7735	0.6667	
AoxD234	0.8061	0.3387		0.8146	0.9815		0.7755	1.0000	
Average +/- S.D.	0.5704± 0.3549	0.4310± 0.3010	5.5714	0.6049± 0.2843	0.4760± 0.2989	6.2857	0.5634± 0.1577	0.4017± 0.3454	3.5714

Table 2. Expected heterozygosity (H_E), observed heterozygosity (H_O) and the mean number of alleles per locus.

The heterozygosity represents an important index that can give information about the diversity and even about the history of a population. The values of the heterozygosity can range from 0 (absence of heterozygosity) to 1 (significant number of alleles with the same frequency). Thus for the locus LS34 in *H. huso* and for LS39 in *A. ruthenus* we obtained a heterozygosity that is equal to 0, since a fixed allele was highlighted for these loci. The values of the average heterozygosity are correlated in direct proportion with genetic diversity.

The Hardy-Weinberg equilibrium was tested with Genepop v1.2 software. Except the p-value for LS19 and LS39 in *A. ruthenus*, the other loci showed significant departures from equilibrium. In generally, the deviation from the expected values might have several causes like the reduced size of the population, inbreeding or the presence of the null alleles which might lead to a false excess of homozygotes.

Our studies come to complete the data of evaluation and monitoring for the population of sturgeon from the Lower Danube. Thus, the data resulting from the monitoring of the YOYs (Young of the Year) born annually in the Romanian part of the Danube and evaluating the success of the natural recruitment have led to the hypothesis that in case of *H. huso* there is a significant generation born before 1990, which represents the basis of gene pool and which cyclic give birth to new generations with a high number of individuals [31].

5. Molecular studies for genetic diversity evaluation and phylogeny inferring in salmonids from Romania

The native salmonid species from Romania have been characterized only from a morphological point of view [28], but the studies based on molecular aspects are still at the beginning. Among the few studies that included the molecular analysis of salmonid fishes from Romania, the evaluation of the genetic differentiation of salmonids by PCR-RFLP technique [76] and the phylogenetic classification of Romanian salmonid species by using the 16SrRNA and 12SrRNA gene sequences [26] were performed.

Even if the salmonids are a well-studied group of fish, there are still a number of questions pending with regard to their phylogeny and evolution. So, despite the fact that a large number of studies based on both morphological [77] and molecular data [78-80] were performed, there are still different opinions concerning genus-level relationships [26]. Four salmonid species from Romania (*Salmo trutta fario*, *Salmo labrax*, *Salvelinus fontinalis* and *Thymallus thymallus*) were analyzed from molecular point of view using 16SrRNA and 12SrRNA markers with the purpose to position them within the *Salmonidae* family. The biological samples were collected from different rivers from Romania (Dambovita, Bratia, Gilau, Latorita, Cerna, and Nera) and from the Danube Delta. Fragments from 16SrRNA and 12SrRNA mitochondrial genes were amplified with specific primers and sequenced by using Sanger method, *dye terminator* variant. For a more complex phylogenetic evaluation beside the sequences determined from salmonid specimens from Romania, 14 salmonid and 1 osmerid sequences from GenBank were also included in the analysis.

Estimation of phylogenetic relationships was achieved using 16S and 12SrRNAs gene sequences and the concateneted data set, while three methodologies – maximum parsimony (MP), maximum likelihood (ML) and Neighbour-joining (NJ) implemented in PHYLIP v3.68 software were used for phylogenetic reconstructions, in order to compare the consistency of the results produced by different methods.

The phylogenetic analysis revealed that primitive salmonid species such as *Coregonus lavaraetus* and the representatives of genus *Thymallus*, *T. thymallus* and *T. articus* occupy basal divergence in the tree topology confirming that the *Coregoninae* and *Thymallinae* subfamilies arise from a common ancestry before *Salmoninae* (with the genera *Salmo, Oncorhynchus, Hucho, Brachymystax* and *Salvelinus*). Based on morphological and molecular data, *Coregoninae* and *Thymallinae* were thought to be the earliest branches within the *Salmonidae* family [80].

The species of the *Salmo* genus form a distinct clade, in which the Atlantic salmon, *Salmo salar* occupies a basal divergence. The data reveal a close relationship between *Salmo trutta fario* and the clade formed by sea trout *S. trutta trutta* and the Black Sea trout *S. labrax*. The resulting clade (*S. trutta trutta, S. labrax*) is not surprising, taking into consideration some characteristics of the life history and reproductive behavior of these species [8].

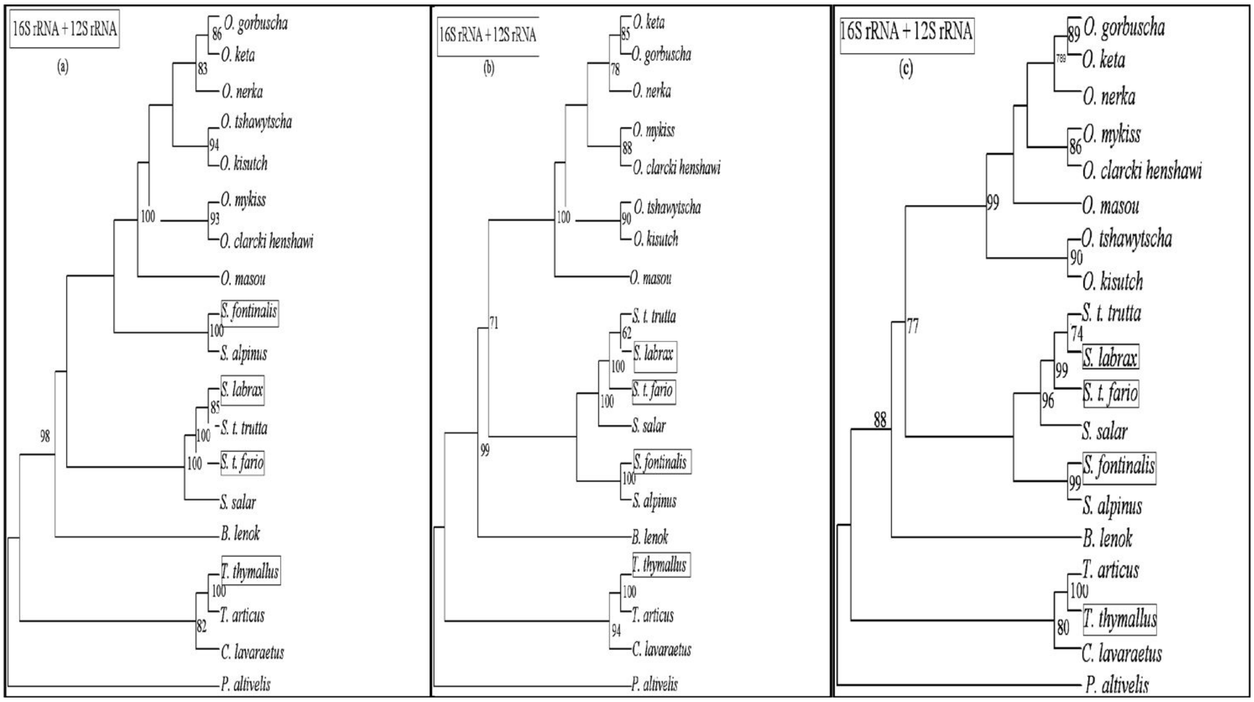

Figure 5. Majority with bootstrap support consensus trees for combined data (16SrRNA and 12SrRNA). (a) Combined data Neighbor Joining tree, distance model Kimura 2 Parameters, transition/transversion ratio 2.3; (b) combined data Maximum Parsimony tree; (c) combined data Maximum Likelihood tree [26].

The monophyly of *Salvelinus* was supported by 16SrRNA, 12SrRNA and combined data, but the position of the clade formed by (*S. alpinus, S. fontinallis*) in relationship with *Salmo* and *Onchorhynchus* is dependent on the molecular marker selected for the phylogenetic analysis. The trees resulted for 16SrRNA and 12SrRNA concatenated data has demonstrated a closer relationship between *Salvelinus* and *Oncorhynchus* than between *Salmo* and *Oncorhynchus*. The relationship between *Salvelinus* and *Oncorhynchus* was also supported by previous molecular phylogeny studies based on three other genes (GH1C, VIT and ND3) [80, 81].

The phylogenetic analysis using mitochondrial ribosomal genes as markers has allowed for the classification of salmonid species from Romania within the *Salmonidae* family. Thus, Romanian *S. trutta fario* and *S. labrax* are placed together within the *Salmo* genus. The *S. labrax*, endemic in the Black Sea, appears to be the sister taxa of the sea trout *S. trutta trutta* from the northwest of Europe (Atlantic coast) and Baltic Sea. The basal divergence in phylogenetic trees occupied by *T. thymallus*, a primitive species, is in agreement with the taxonomic and evolutionary data. Unfortunately, the position of the *Salvelinus* genus relative to the *Salmo* and *Oncorhynchus* genera remains controversial. The data reveal a possible sister taxon relationship between *Oncorhynchus* and *Salvelinus* despite the fact that morphological data support a closer relationship between *Salmo* and *Oncorhynchus*, thus confirming earlier findings of Crespi & Fulton [80] and Oakley & Phillips [81].

Unfortunately, salmonids' natural habitat is disrupted by a series of human activities such as poaching, dams, ballast exploitation and the construction of the micro hydro plants on the water streams from mountain areas. In this context, a preliminary study was directed to towards the assessment of the anthropic impact on the brown trout (*S. trutta fario*) populations from different rivers in Fagaras Mountain, including beside ecological aspects, molecular ones [82]. The area of Fagaras Mountain is the most representative in Romania for the brown trout populations, the tourism and recreational fishing being well known in this area, while the "brown trout of Fagaras" is preferred for alimentary consumption.

In the proposed study 102 individuals of brown trout from four populations from the Meridional Carpathians (Arpas, Ucea and Sambata rivers in the Northern versant of the Fagaras Mountain) were analyzed using mtDNA marker (D-loop) and nuclear markers (nine microsatellite loci - Str73, Str15, Str60, OmyFgt1, Ssa197, Ssa85, Strutta12, Str543, BS131).

The data obtained by sequencing of the complete mitochondrial control region (D-loop) were compared with similar sequences from GenBank. The phylogenetic tree resulted from analysis with MEGA v.5 contained, besides the sequences of D-loop in *S. trutta fario* individuals sampled in the Romanian rivers, complete D-loop sequences from other European lineages in order to observe the affiliation of analyzed Romanian individuals. The dendogram topology showed a classification of analyzed sequences in distinct monophiletic groups corresponding to each evolutive lineage and that the representatives of brown trout from Romania analyzed in the study were placed in the Danubian clade similar to other sequences selected from GenBank and belonging to Danubian lineage (Figure 6).

In Romania, Danubian lineage of brown trout is native and by founding the specific haplotypes for this lineage in our individuals, means that either restocking programs have not been done yet in the area or, if they have, the individuals involved were selected properly [82]. This type of approach can be really useful when the data about restocking and management of the rivers are poor or completely missing.

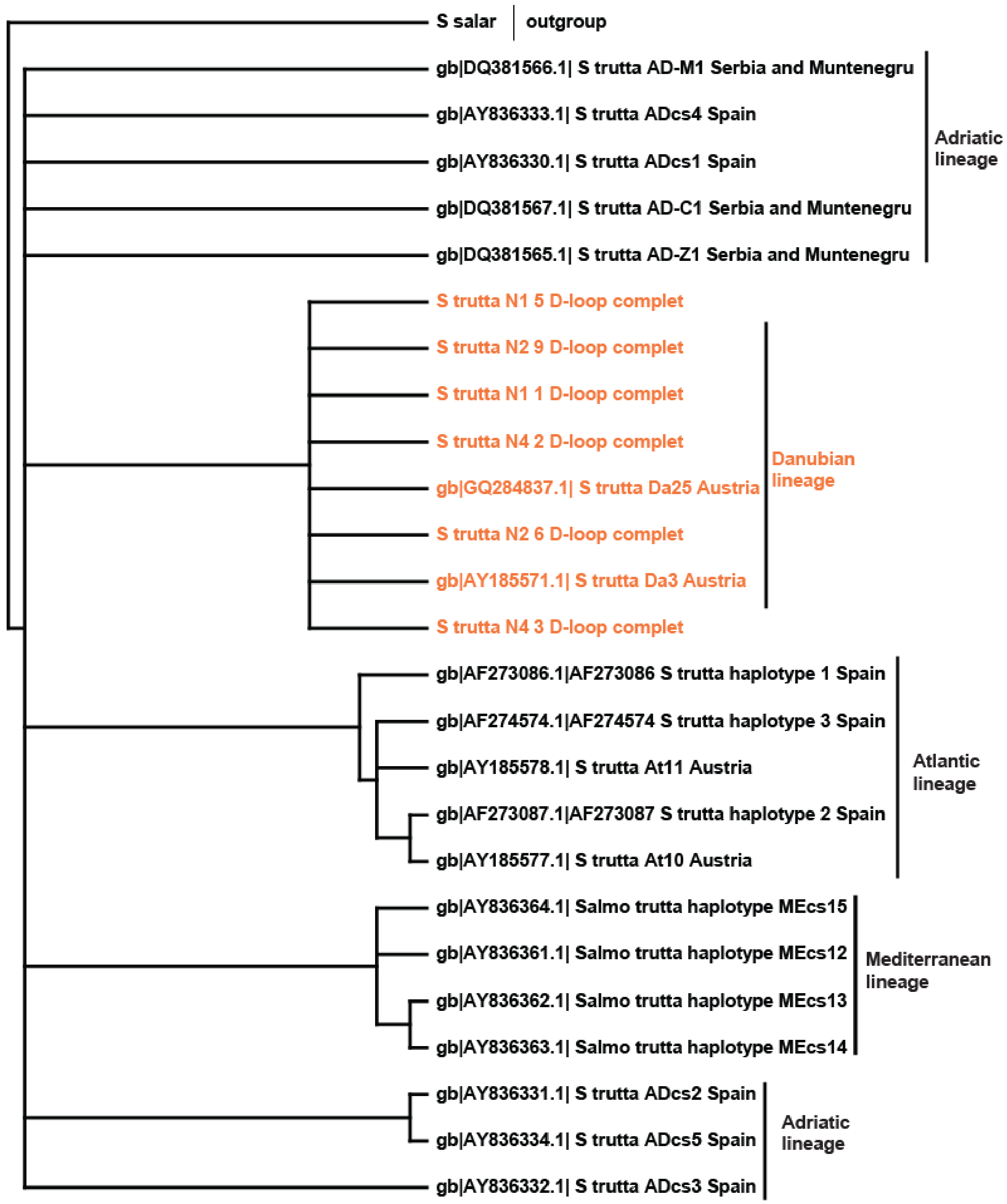

Figure 6. Phylogenetic tree obtained with MEGA v5 (NJ method, bootstrap value 1000 replications) illustrating the relationships between the evolutive lineages of brown trout. The haplotypes from Romania are placed in the Danubian lineage along with two sequences from GenBank belonging to the same lineage [82].

The genetic differentiation between the four population of brown trout analyzed in this study was evaluated by F-statistics, by using the indices F_{st} and $Gamma_{st}$ calculated with DNAsp software. F_{st} (fixation index) is a Wright statistic index that indicates the genetic variation between the populations and that can take values from 0 to 1. In generally, a higher value than 0.25 is correlated with a high genetic differentiation between populations. $Gamma_{st}$ is similar to F_{st}, making a correction for insufficient sampling. The values obtained for the four populations of brown trout indicate that there is a high differentiation between them from genetic point of view (Figure 7).

Population 1	Population 2	GammaSt	Fst
N1	N2	0.59009	0.72259
N1	N4	0.39013	0.55081
N1	N3	0.44519	0.58468
N2	N4	0.19504	0.30877
N2	N3	0.12098	0.17850
N4	N3	0.18104	0.28120

Figure 7. F_{st} and $Gamma_{st}$ values for four populations of brown trout from Romanian rivers [82].

The microsatellite data confirmed the previous results obtained regarding the genetic differentiation. Based on genotypic data resulted consequently microsatellites analysis a Factorial Correspondence Analysis (FCA) was performed by using GENETIX software. This type of analysis assumes the applying of a multidimensional method that permit the conversion of genotypic data obtained by the analysis of the nine microsatellite loci characteristic to each analyzed individual in points distributed in an X, Y, Z axis system. Thus, consequently to this analysis four different clusters were obtained corresponding to the four populations of brown trout, indicating a genetic differentiation between the analyzed populations (Figure 8).

The value of F_{is} index in the analyzed population indicates is positive for each population and indicates a light level of inbreeding within the population (Table 3).

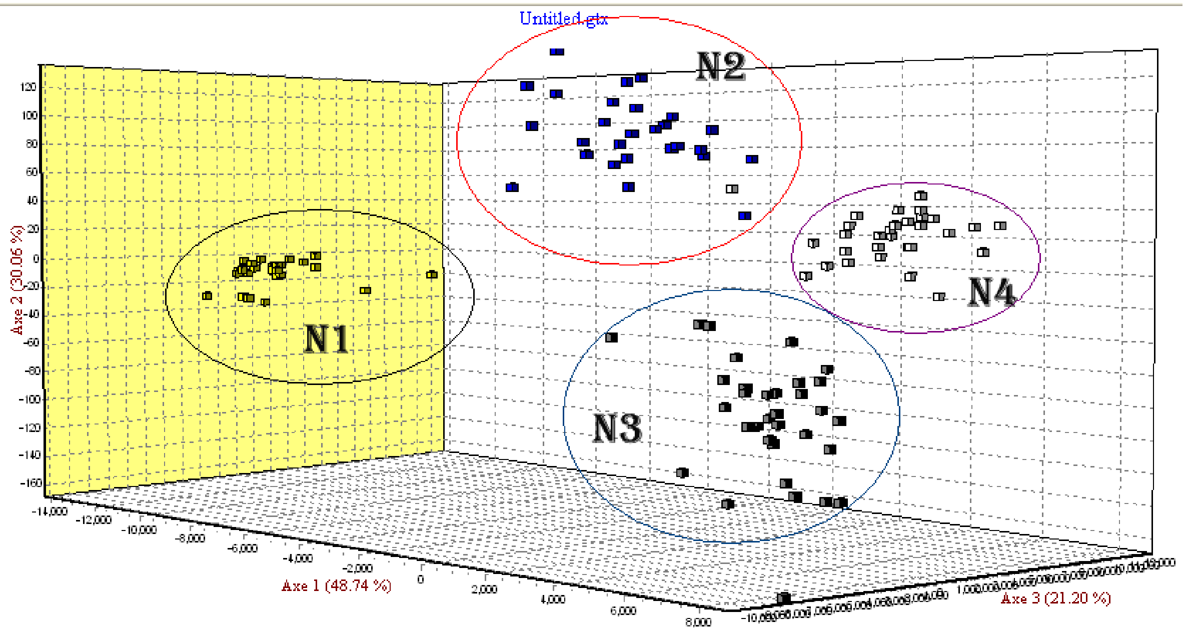

Figure 8. Factorial Correspondence Analysis for four populations of brown trout from Romanian rivers.

Locus	Population N1	Population N2	Population N3	Population N4
Str60	0.000	-0.250	0.477	-0.747
Str15	0.618	-0.190	NA	0.444
Str73	NA	NA	NA	NA
OmyFGT	0,143	-0.052	0.224	0.179
Ssa85	-0.500	NA	NA	0.505
Ssa197	0.191	0.254	0.017	0.094
Str543	0.020	0.911	-0.053	0.209
Strutta12	0.094	0.358	0.153	0.399
BS131	1.000	0.259	0.253	-0.127
Average	0.094	0.270	0.139	0.134

Table 3. F_{is} values calculated with FSTAT software. NA (not assigned).

Concluding, the analyzed populations are natives (Danubian lineage), differentiated by genetic point of view, but with a light level of inbreeding within population which might conduct in time to a reduced genetic diversity.

The studies are intent to be extended first to the Southern side of Fagaras Mountain, which is considered to be the most affected of the anthropic intervention, then to the main basins in the country populated with brown trout. The information resulted from these studies can be further used in programs of management and conservation, with accent on keeping unmodified the autochthonous species.

6. Conclusions

The studies developed in our country for analyzing in terms of genetic diversity species and populations of great scientific, ecological, economic and social importance are still at the beginning.

The molecular analyses of Lower Danube sturgeons were directed to species identification and assessing of genetic diversity. Regarding the first topic, a method based on microsatellite markers was set up as it was proven that the identification of species based only on morphology and mtDNA analysis can be misleading and do not serve to hybrid detection. The populations of sturgeon from Lower Danube appear to be fragile from genetic diversity point of view, but the studies should be extended in a higher number of individuals and using a more significant and informative set of molecular markers

The analysis of microsatellites in these species is complicated by the complexity of the genome, so a better selection of a much significant number of loci with disomic inheritance pattern is recommendable. The decision to initiate restocking programs in order to recover the natural sturgeon populations must take into account not only the social and economic aspects, but also the assessment of genetic diversity.

The studies on salmonid were focused on mainly on phylogenetic classification of species from Romanian fauna. The research aiming on genetic diversity analysis is still in an incipient phase and was directed towards the populations of brown trout from Fagaras Mountain, an important area for salmonid distribution in our country. The results of these studies based on mtDNA and microsatellites analysis showed that the studied populations are pure Danubian brown trout lineage and genetically distinct. In the future, for a better image of salmonids status in Romania the research would be extended in analysis populations from other salmonid species and the area of sampling would be enlarged to the entire Romanian Carpathian Arch.

7. Perspectives

As our studies are in the preliminary phase, in future we intend to extend them in a higher number of individuals/populations from both fish groups consider of being of highly importance for our country.

Also, we intend to characterize from genetic point of view populations/ stocks/ strains from aquaculture, as we consider that exploitation of genetic data is imperious necessary beside restocking and conservation, for genetic improvement in aquaculture.

Currently the microsatellites and mtDNA are considered to be classical markers. There is an increasing tendency of analyzing the nucleotide variation at the whole genome level by *Next Generation Sequencing (NGS)* techniques. This type of approach started to be applied for different fish species analysis, including sturgeons and salmonids. NGS variants as *RAD (Restriction Sites Associated DNA) sequencing* are used for identification and characterization of a complete panel of SNP markers consider to be extremely useful for genomic analyses at individual, population and species level. This type of markers appears to be highly informative and of real support for conservation programs as is the trend of passing from the conservation genetics to conservation genomics era. The identification by NGS of an extended panel of specific markers should be extremely useful for sturgeons from the Lower Danube in population management and conservation purposes. The analysis should be orientated toward *A. gueldenstaedtii* the most affected sturgeon species from the Danube. This is a polyploid species, presenting a complex genome structure, fact that makes very difficult the analysis using classical markers. Also, the setup of such complex panel of SNP markers would make possible the traceability of caviar in the sturgeon species from Romania.

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