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Corso di Laurea Magistrale in BIOLOGIA MARINA

Reproduction and artificial restocking of *Acipenser naccarii* (Bonaparte, 1836). Genetic and physiological characterization through microsatellite markers and radioimmunoassay of sexual steroids

Tesi di laurea in Genetica e Biotecnologie in Acquacoltura

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ABSTRACT

The Adriatic sturgeon, *Acipenser naccarii* (Bonaparte, 1836), is a highly threatened species due to human activities, particularly overfishing and habitat destruction. Its peculiar ecology and biology (restricted areal and anadromy) makes this species particularly vulnerable. In March 2010 the IUCN has identified the Adriatic sturgeon as a critically endangered species according to the Red List of Threatened Species. Due to its rapid decline, starting from the 80s, at present there is no evidence of natural reproduction in wild environment, which makes the Adriatic sturgeon dependent on captive breeding programs that need to be improved in order to be effective for the survival of the species. For this purpose this study aims to characterize artificial restocking population of Adriatic sturgeon, with both genetic and physiological analysis in order to establish an efficient restocking program for future reproductions. The research is structured on two levels: First genetically, by analyzing 9 microsatellite loci. This gives information relatively about parent allocation and kinship between individuals that were sampled for this study. Hence to predict which reproduction events are the most optimal in terms of incrementing genetic diversity, by the estimation of multilocus pairwise band sharing coefficients. Second step, physiological analysis: testosterone (T) concentration levels in each individual were measured for sexing, without sacrificing the lives of the animals with the use of an invasive examination of the gonads. The combination of interdisciplinary analysis is important to obtain an overall picture in order to indicate the main broodstock participating in reproduction events and future optimal potential participants, in order to ensure a valid management for restocking program and their monitoring.

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1.1 Taxonomy and geographical distribution

Fig.1 Specimen of *Acipenser* naccarii (www.entetutelapesca.it).

PHYLUM	*CHORDATA*
SUBPHYLUM	*VERTEBRATA*
CLASS	*OSTEICHTHYES*
SUBCLASS	*ACTINOPTERYGII*
INFRACLASS	*CHONDROSTEI*
ORDER	*ACIPENSERIFORMES*
FAMILY	*ACIPENSERIDAE*
SUBFAMILY	*ACIPENSERINI*
GENUS	*Acipenser*
SPECIES	*Acipenser naccarii*

The Adriatic sturgeon (*Acipenser naccarii*, Bonaparte 1836) is classified under the order of the Acipenseriformes, infraclass Chondrostei, that is dated phylogenetically at least 200 Mya during the Jurassic period (Patterson, 1982). They represent one of the most ancient fish groups, which have successfully survived several mass extinction events during their history and have conserved morphological ancient features, such as an endoskeleton for the most part still cartilaginous. In fact they are known as “living fossils”, also for their peculiar genetic state.

Acipenseriformes are composed of 25 sturgeon species and two paddlefish species that are distributed exclusively in the Northern hemisphere (Dudu, 2011). This order contains two monophyletic families: Polyodontidae and the

Acipenseridae. The Acipenseridae family is divided into three subfamilies: Husinae (Findeis, 1997), Acipenserinae, including Scaphirhynchini (Grande and Bemis, 1997) and Acipenserini.

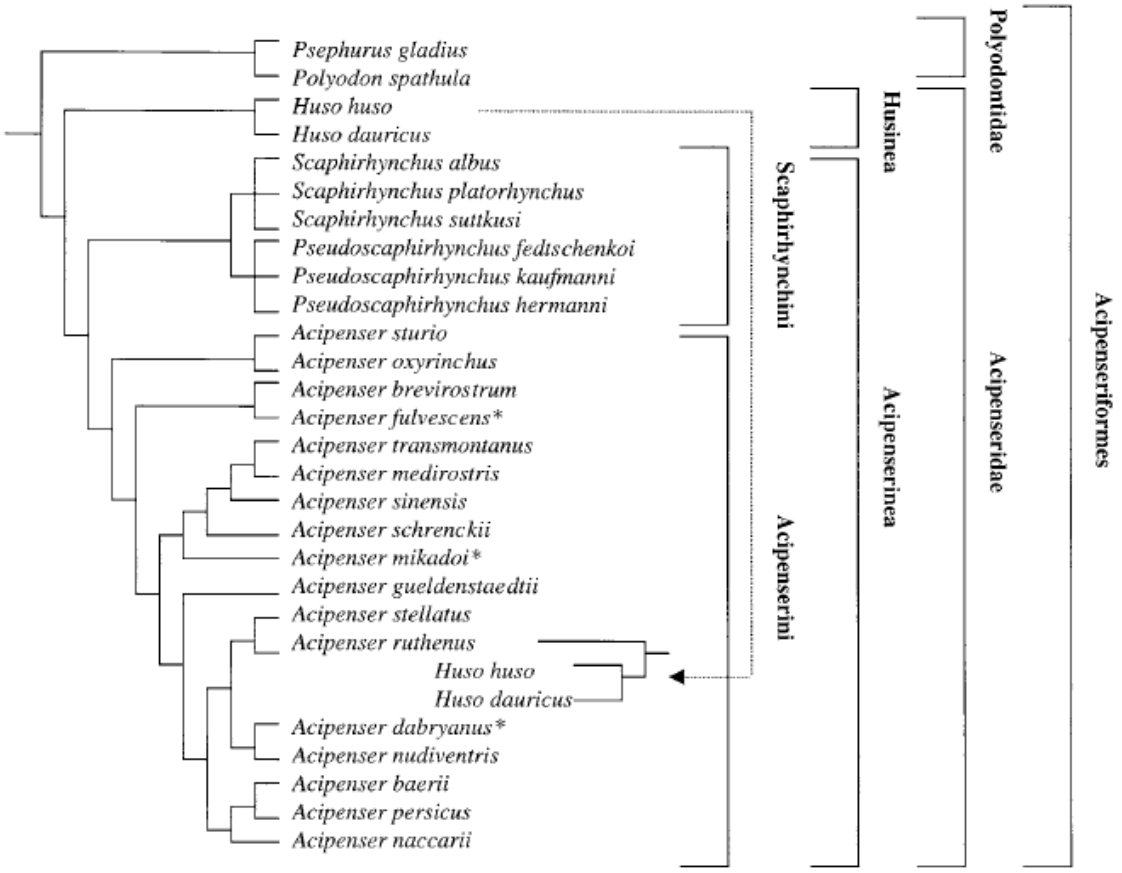


Fig.2 Interrelationships of extant Acipenseriformes (Bemis and Kynard 1997).

The taxonomic criteria mostly used for phylogenetic studies are based on morphological and ecological aspects, but also on the biogeographical distribution of the species.

Bemis and Kynard (1997) conducted different biogeographic analysis of living Acipenseriformes on 85 rivers in witch spawning occurs, and pointed out 9 biogeographic provinces (Billard *et al.,* 2001). The provinces are: North Eastern Pacific, Great Lakes, North Western Atlantic, Mississippi-Gulf of Mexico, North Eastern Atlantic, Ponto-Caspian and other local seas, Siberian and Arctic Ocean, Amur River Basin, Chinese, Japanese and Okhotsk Seas. The possibility of exchanges between provinces is very limited, but the same species can be found in different locations.

Although there is a significant number of data, thanks to the rising scientific interest for this monophyletic fish group, the demonstration of the origin and the relationships between different species, are still unknown. This is due to the high

morphological variability of sturgeons and to many intraspecific hybridization events, with prolific offspring, that have been occurred during time even between species from different genera (Fontana *et al,* 1999).

The Adriatic sturgeon was historically spread in Italy, Albania and former Yugoslavia (Crivelli, 1996). More specifically in Italy it was distributed in the high Adriatic Sea and Po River and its tributaries. It was recorded in the rivers Adige, Brenta, Bacchiglione, Livenza, Piave and Tagliamento. In Italy the wild population is most likely extinct as there is no evidence of spawning from wild individuals, the last spawning occurred is registered in early 1980s. Data collected between 1995 and 1997 reveal the presence in the Skadar Lake and river Buma, border between Albania and Montenegro (Williot *et al*., 2002). Recent studies support the contention that historical distribution of this species includes certain Spanish rivers (De La Herran *et al*., 1999).

Fig.3 Distribution of *Acipenser naccarii* in Europe (www.ittiofauna.org).

1.2 Morphology and distinguishing features

The morphology of sturgeons in general, is highly characteristic. They show elongate body with a ventral flat base, the presence of scutes, that are bony dermal plates located longitudinally in rows along the body. Other peculiar features are: heterocercal caudal fin, rostrum, ventral mouth, barbels, gill-rackers, notochord, intestinal spiral valve and a well-developed spiracle or vent-hole. These general descriptive features can be considered a mix of primitive and derived characters that can also be found outside Acipenseriformes. Although some features could be mistaken for primitive they are actually the result of regression, such as ossification reduction and the absence of teeth in adults (Bemis *et al.*, 1997).

It is common to confuse *Acipenser naccarii* (Fig.4.) with other species, such as *Acipenser gualdenstaedtii* (Fig.5.), *Acipenser persicus* (Fig.6.) and sometimes with *Acipenser sturio* (Fig.7.), so it is important to perform an accurate visual investigation of the distinguishing features.

Fig.4 Specimen of *Acipenser naccarii* (www.arkive.org).

Fig.5 Specimen of *Acipenser gualdenstaedtii* (www.ittiofauna.org).

Fig.6 Specimen of *Acipenser persicus* (www.sturgeon.ir).

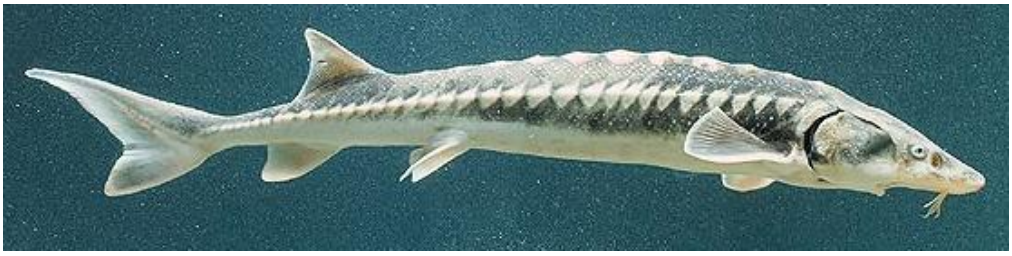


Fig.7 Specimen of *Acipenser sturio* (www.arkive.org).

The Adriatic sturgeon shows an elongated body with the head that covers 20-22% of total length (Forneris *et al*., 1990). The rostrum is wide and short in adults, but shovel shaped in juveniles. The barbels origin closer to the tip of snout rather than the mouth. The body is covered with prominent dermal denticles between the principal rows of scutes. They show a defined number of scutes: 9-21 mid dorsal, 29-42 lateral, 8-11 ventral (Hochleithner *et al.*, 2012). In juveniles the denticles are tighter than adults and give the impression as to form rhombic platelets (Bronzi *et al.*, 2005). Usually is present a single large pre-anal plate. Unlike most Acipenser the posterior anal fin margin dose not reach the origin point of the lower lobe of the caudal fin. The dorsal fin origins behind the anus. The livery can vary, it is generally grey or brown and the ventral side is white. Juveniles show prominent black saddle markings on body.

1.3 Ecology and Biology

The Adriatic sturgeon is an anadromous species (migrating up rivers from the sea to breed in fresh water). It can generate populations that can complete their entire reproductive cycle in fresh water. The adult's favourite habitat consists in sandy or muddy river bottom at 10-40 m of depth (Hochleithner *et al.*, 2012). While staying in rivers, the Adriatic sturgeon seeks for pools with high flow rates, however, when in sea, it prefers staying along the coastal area. The diet consists mainly in benthic invertebrates, such as: insect larvae, crustaceans, molluscs and worms, but also small fish and organic debris from various sources (Porcellotti, 2005). This species feeds both at sea and in fresh water and are dependent on the quantity and quality of prey available.

The life cycle of Acipenseriformes in general is quite long reaching puberty stage late in life. The adult male of *Acipenser naccarii,* reaches sexual maturity at 6-7 years, to a length of about 70 cm, while females growth is slower and do not reproduce until they reach 8-12 years of age and 80-120 cm length (Hochleithner *et al.*, 2012). The reproduction occurs with an annual pace for the males, instead the females spawn every 2 to 3 years due to the huge energetic investment that lead to the production an amount of eggs that is equal to 20% of the total body weight (Tortonese, 1989). Spawning takes place along the banks of the rivers, where water flow is quite low, and occasionally in brackish water. The eggs have a diameter of 2.2 to 3.3 mm and hatch in 150-180 hours after spawning, with water temperature rates of 15-16°C. In spite of high egg

production per spawning by a female sturgeon, late sexual maturity and long breeding intervals contributes to low overall reproductive rates and make them particularly vulnerable to environmental changes in their reproductive habitats (Doroshov *et al.*, 2011). The growth of larval and juveniles stages occurs generally in fresh waters. In autumn after reaching one year of age, the young sturgeons migrate towards the sea and return to rivers only when they reach complete sexual maturity when they reach an average size of 20 kg for male and 30 kg for female. The maximum size that an Adriatic sturgeon has reached, according to current bibliography, is between 150 and 200 cm length approximately and a weight up to 25-30 kg (Forneris *et al*., 1990). From our experience a mature species can measure far more than 200 cm when in optimal conditions.

Nowadays the natural reproductive cycle is not carried out anymore, the only known populations are “landlocked” and their migratory habits have been interrupted leading to a substantial variation from the biological point of view.

1.4 Physiology and sexual differentiation

It is important to understand the physiological response to the effects due to changes of environmental conditions such as: temperature, dissolved gases and salinity causing physiological response to hypoxic, hypercalcemic and saline environments. These species are particularly sensitive to small variation of the external conditions that could seriously compromise their performance level (reproduction, growth and locomotion) and redirecting energy form growth or reproduction to other energy-conservation categories. Also understanding the time frames and limits of these responses and their potential costs may help to meet conservation, management and protection goals for a better use of sturgeon resources.

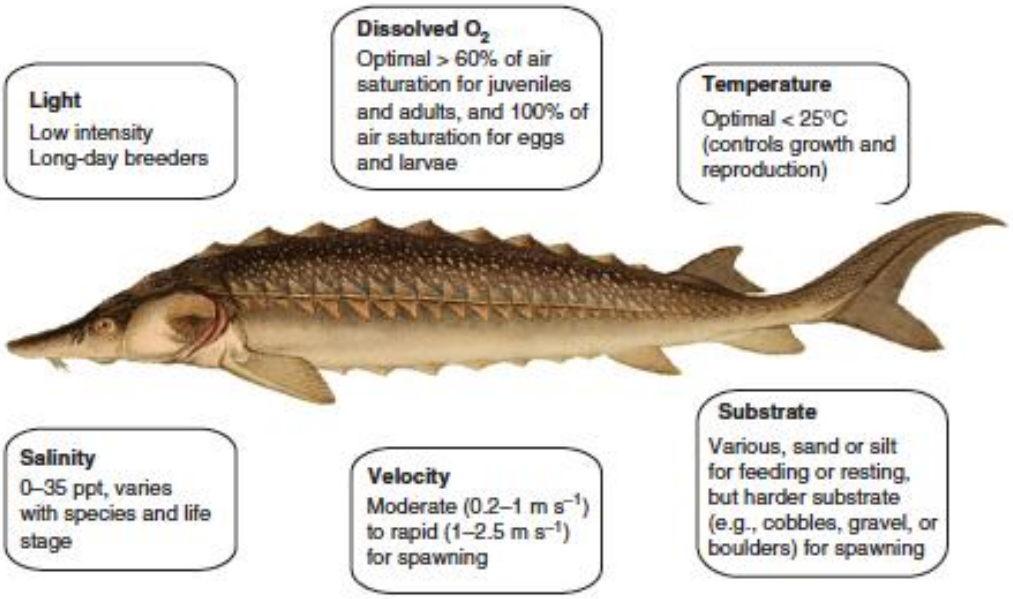


Fig.8 Schematic environmental requirements for sturgeon (Doroshov *et al*., 2011).

In vertebrates, sexual differentiation occurs with the development of undifferentiated gonads and their transformation in testicles and ovaries. This produces the phenotypic sex of the individual that contributes to the sex ratio of the population. Puberty, the onset of the reproduction capacity, in sturgeons is recognized when the amount of concentration of sexual steroids in the plasma reaches a certain level that can induce proliferation of spermatogonia in males, and the start of vittellogenesis in females (Doroshov et al., 2011). At this certain stage the energy normally used for growth is spent for reproduction. Pubertal age varies from 5 to 15 years in wild life, but farmed sturgeon show early puberty (3-6 years) due to continuous feeding, on a high energy diet and growth to high temperature (18-24 °C). The onset of puberty is due to both environmental signals, such as photoperiod and temperature, and secretion of two pituitary gonadotropins: FSH (follicle stimulating hormone) and LH (luteinizing hormone) that regulate the reproductive cycle of the sturgeon. Plasma concentration of FSH is elevated during gonadal growth and vitellogenesis, whereas the plasma concentration of LH rises during spawning season.

The system is regulated by a complex feedback pathway, which involves the hormones of the hypothalamic-pituitary-gonadal reproductive axis (BGP) (Shulz *et al.,* 2011). The GnRH, (gonadotropin – realising hormone), stimulates secretions of FSH bad LH that control the synthesis of gonodal sex steroids such as androgen, estrogen and maturation-including steroids (MISs). Sex steroids are synthesised in the ovarian theca, granulosa cells and testicular Leydig cells. In

sturgeon gonadal steroids are testosterone (T), 11-ketotestosterone (11-KT), estradiol-17 β (E_2) and MIS 17,20β-dihydroxy-4-pregen-3-one (17,20β-P). All these hormones circulate in both sexes at different concentration levels and exert different activities (Doroshov *et al*., 2011).

Testosterone provides the substrate for 11-KT, that is implicated in controlling spermatogenesis; it also provides E_2 that stimulates secretion of the yolk precursor in the liver and maintenance of ovarian follicles in the late phase of oogenesis, and has a positive feedback for sturgeon pituitary gland.

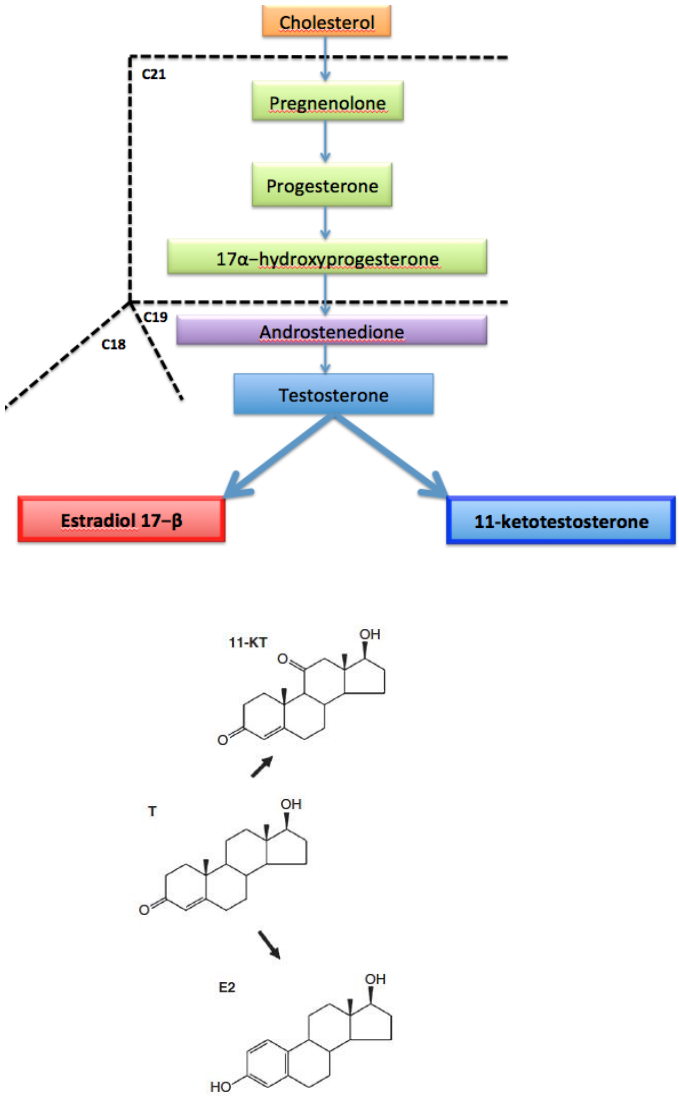


Fig.9 Sex steroids production starting from cholesterol (on top); molecular structure of T, E2 and 11-KT (Shultz *et al.,* 2011).

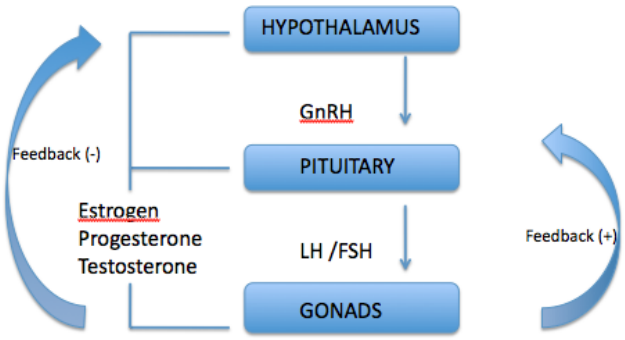


Fig.10 Schematic representation of the endocrine system regulating reproduction, the BPG axis. GnRH produced by the Hypothalamus interacts with its receptors on the two gonadotropic cell types of the pituitary, which produce either LH or FSH that control the synthesis of gonodal sex steroids. Sex steroids hormones feed back to the brain-pituitary system.

1.5 Genetics

A distinctive feature of the Adriatic sturgeon is its genomic complexity that can show different levels of ploidy (Fontana *et al.*, 2008). Studies based on the analysis of sturgeon karyotype have revealed two main groups of species that present different number of chromosome: diploid, with 120 and tetraploid, with 240, respectively. Although the level of ploidy to be ascribed to these chromosome numbers is still being debated.

The Adriatic sturgeon belongs to a cluster of four 240 chromosome species, including *Acipenser persicus* (Persian sturgeon), *Acipenser gueldenstaedtii* (Russian sturgeon) and *Acipenser baerii* (Siberian sturgeon). The high number of chromosome was likely occurred due to multiple polyploidization events starting from a 60-chromosome common ancestor (Fontana *et al.*, 2007). Focusing on the origin of tetraploidy, all the tetraploid species in the northern hemisphere could be either auto-tetraplodids (derived from an interspecific genome duplication) or allo-tetraploids (when tetraploidy is reached throughout hybridization events). This particular state is due to different segregation modalities of chromosome into gametes and the product of the segregation can be either tetrasomic or diasomic (Stif *et al.*, 2008). The tetrasomic pattern is followed when each chromosome shows four homologous copies, and each copy can randomly pair with any other homolog during meiosis and the six possible combinations all can segregate into gametes. A diasomic inheritance is slightly different

and it occurs when four homologs are present that cannot randomly pair up during meiosis. So the result of segregation is only four different possible allele combinations, instead of six. Besides these two different modalities of chromosome segregation into gametes, it is possible that different degrees of pairing between chromosome can occur and lead to intermediate inheritance patterns where it is possible to expect all the possible combinations but with different frequencies (Stif *et al.*, 2008). These data are significantly important not only because they provide information on the origin of their karyotype, but help to understand and predict the inheritance pattern in tetraploid sturgeons, in order to establish a correct management of breeding programs and monitoring the residual genetic variability for both conservation aquaculture (Rodzen *et al.,* 2002). In order to fulfil a correct long-term breeding plan it is necessary to apply reliable parental allocation procedure, and especially on a tetraploid species it cannot neglect the modality of inheritance. The genotypes that are expected by the progeny of a given parent vary under different transmission models. It is also very important to know the inheritance patterns for developing virtual offspring simulation procedures that can be useful for the assessment of the expected range of genetic variation, based on different degrees ok kinship (Congiu *et al.*, 2011). Little we know about inheritance models of sturgeons, in fact the only available studies were conducted on two tetraploid species from North America: *Acipenser transmontanus* (Rodzen and May, 2002) and *Acipenser fulvescens* (McQuown et al., 2002; Pyatskowit et al., 2001).

1.5.1 Microsatellite markers

Microsatellites are tandemly repeated motif that vary from 1 to 6 nucleotides, and are also known as simple sequence repeats (SSRs) or short tandem repeats (STRs). These motifs are usually non-codifying and reflect a Mendelian model of inheritance. They also have a characteristic mutational behaviour and because of this they are typically highly polymorphic (Ludwig, 2008). The alleles (fragment length) at a specific locus can defer at number of repeats and the variation length is used for the definition of the allele and its detection is PCR-based. Because microsatellites profiles vary among specimens and are widely distributed in the genome, they are used in many genetic research areas (Ludwig, 2008).

In aquaculture, for the association between progeny and broodstock (Parental allocation), fingerprinting is needed and microsatellite are mainly used for this purpose due to their polymorphic and codominant characteristics (Galli *et al*., 2011). Also an

artificial selection of the genotypes is required to establish a valid restocking programme, this is possible by applying a Marker Assisted Selection (MAS) with the use of microsatellite loci (Lande et al., 1990).

1.6 Protection measures for the species

1.6.1 Threats

The peculiar ecology and biology of the Adriatic sturgeon (restricted areal and anadromy) makes this specie particularly vulnerable to fast environmental changes due to increasing anthropization and unsustainable use of natural resources. The causes for the decline of *Acipenser naccarii* over the time, are different and are the result of the combination of several actions. The major threats are:

- Overfishing, both legal and illegal;
- Creation of barriers to its migratory routes, which reduces its reproductive success, that is now limited by the fact that spawning does not occur in captivity;
- Fragmentation of populations, particularly by the building of dams for hydropower;
- Competition for habitats with allochtonus species, *Silurus glanis*;
- Water pollution;
- Allee effect.

In Italy, up to 1987, the minimum size legal catch was fixed to 60cm of length, which focalized the fishing effort mostly on the juveniles in pre-reproductive age: over 80% of 2000 specimens that were sold to the fish market during the period between 1981 and 1988, did not exceeded 3,5 Kg in weight. Inevitably, overexploitation of specimens, that have not reached sexual maturity, affects negatively growth and genetic variability, leading to a much serious condition in time that is extinction of the species. In 1920 the annual catch of the Adriatic sturgeon was about 100 tons, in 1950 it reduced to 15 tons to decrease drastically up to 2 tons between the 70s and 80s (Hochleithner *et al,*. 2012).

Data collected on fishing activity (Kg per year) in the Po River, from early 80s to early 90s (Fig. 11) show a clearly decreasing trend of catches of wild species.

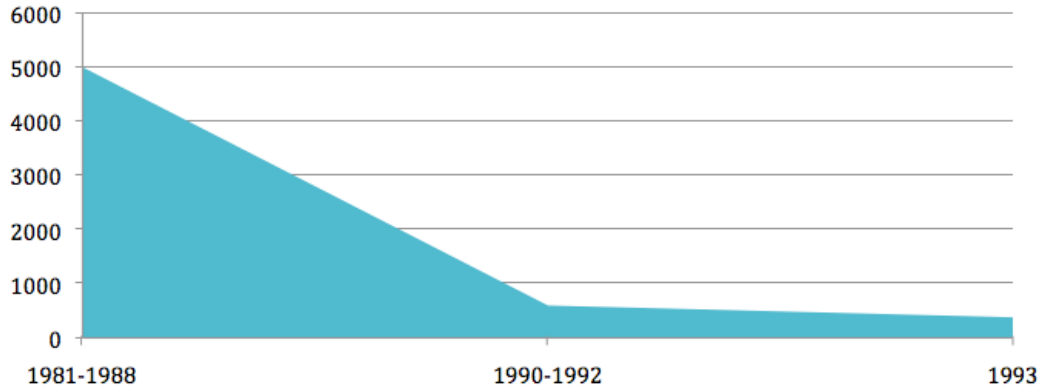


Fig.11 Graphic showing decreasing trend of catches of *Acipenser naccarii* in wild life.

Recent data collected on trade of the top ten species of fish exported in Italy (Fig.12), (CITES trade data dashboards) in a range of time going from 2007 to 2010 show how the genus Acipenser in general is highly exported and the numbers of live species of *Acipenser naccarii* is significantly high as well.

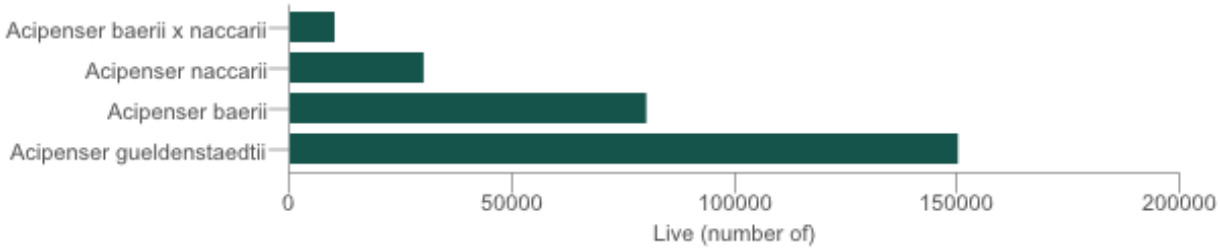


Fig.12 CITES trade data dashboard showing the top 10 species exported in Italy (www.cites.org).

Another threat is represented by the creation of barriers to its migratory routes, which reduces its reproductive success, can cause fragmentation of population and also can lead to irreversible changes of the biology: from anadromy to landlocked species. An example is given by the building of the dam of Serafini's island in Piacenza, created for hydropower purpose, on the Po River, and the dam created in the Drin River upstream of which has established a landlocked population (Ludwig *et al.*, 2003).

1.6.2 Conservation status

The Adriatic sturgeon, in its present status, is extinct in most of its original range of distribution (Forneris *et al.*, 2012). Latest data, about natural spawning, date back to the early 80s. For this reason, the species is now protected by EU laws, which prohibit capture and killing and is subjected to recovery programmes (Porcellotti, 2005). The Adriatic sturgeon is considered by the Directive 92/43/EEC, as one of " the species, animals and plants, of community interest whose conservation requires the designation of special areas of conservation" (annex. II), and is among the "species that are in need of strict protection" (annex. IV). *Acipenser naccarii* appears also in the list of specially protected species in the Bern Convention (Appendix. II), and is reported in Annex B of the Community regulation on trade in wild fauna and flora in compliance with the Washington Convention (CITES) (Zerunian, 2002). This specie is also included in the "Red list" on the conservation status of the species of the International Union for Conservation of Nature (IUCN) and is classified as "critically endangered": extremely high risk of extinction in the wild (www.iucnredlist.org).

In Italy, since the 80s, the first experiments were conducted on artificial insemination and captive breeding (Fornersi *et al.*, 1990) with the aim to obtain material for restocking programmes. The first specimens released, with different growth stages, where in Lombardy in the 90s in the rivers Ticino, Adda and Oglio and from 1999, in Veneto in the rivers Piave, Livenza and Sile (Zerunian, 2002).

1.6.3 International protection measures

1.6.3.1 Directive 92/43/EEC

The species *Acipenser naccarii* is listed in Annex II of the Council Directive 92/43/EEC, also known as Habitats Directive, of May 21st 1992, on the conservation of natural habitats and of wild fauna and flora of community interest whose conservation requires the designation of special areas of conservation. The Habitats Directive, together with the Birds Directive 2009/147/CE, forms the corner stone of Europe's nature conservation policy. It is built around two pillars: the "Natura 2000" network of protected sites and the strict system of species protection. In all, the directive protects over 1.000 animals and plant species and over 200 so called "habitat types" (e.g.

special types of forests, meadows, wetlands, etc.), which are of European importance (www.ec.europa.eu).

Acipenser naccarii is among the species that the Habitats Directive defines a "priority", which needs special and fast intervention for the safeguard and recovery of the species. It is also included in the Annex B, between species for which conservation is necessary to designate special areas of conservation, and in Annex D, the most in need of intervention and support measures for their protection.

1.6.3.2 Bern Convention

The Bern Convention on the Conservation of European Wildlife and Natural Habitats, also known as the Bern Convention (or Berne Convention), is a binding international legal instrument in the field of Nature conservation. It covers the natural heritage in Europe, as well as in some African countries. The Convention was open for signature on September 19th 1979 and came into force on June 1st 1982. It is particularly concerned about protecting natural habitats and endangered species, including migratory species. The convention has three main aims, which are stated in Article 1:

- to conserve wild flora and fauna and their natural habitats;
- to promote cooperation between states and to give particular attention to endangered and vulnerable species including endangered and vulnerable migratory species (www.coe.int).

1.6.3.3 Bonn Convention

The objective of the Bonn Convention (CMS), Council Decision 82/461/EEC of June 24th 1982, is the conservation of migratory species worldwide. Wild animals require special attention because of their importance from the environmental, ecological, genetic, scientific, recreational, cultural, educational, social and economic points of view. The Bonn Convention was signed in 1979 in Bonn, Germany and entered into force on November 1st 1983. The parties acknowledge the importance of conserving migratory species, and the need to pay special attention to species the conservation status of which is

unfavourable. To avoid any migratory species becoming endangered, the parties must endeavour:

- to promote, cooperate in or support research relating to migratory species;
- to provide immediate protection for migratory species included in Appendix I;
- to conclude Agreements covering the conservation and management of migratory species listed in Appendix II.

Acipenser naccarii is listed in the Appendix I of the CMS for migratory species (www.minambiente.it).

1.6.3.4 Convention on trade in Endangered Species of Wild Flora and Fauna, CITES

CITES (the Convention on International Trade in Endangered Species of Wild Fauna and Flora) is an international agreement between governments. Its aim is to ensure that international trade in specimens of wild animals and plants does not threaten their survival. CITES was drafted as a result of a resolution adopted in 1963 at a meeting of members of IUCN (The World Conservation Union). The text of the Convention was finally agreed at a meeting of representatives of 80 countries in Washington, D.C, United States of America, on March 3rd 1973, and on July 1st 1975 CITES entered in force. The original of the Convention was deposited with the Depositary Government in the Chinese, English, French, Russian and Spanish languages, each version being equally authentic. CITES is an international agreement to which States (countries) adhere voluntarily. States that have agreed to be bound by the Convention ('joined' CITES) are known as Parties. Although CITES is legally binding on the Parties, they have to implement the Convention, it does not take the place of national laws. Rather it provides a framework to be respected by each party, which has to adopt its own domestic legislation to ensure that CITES is implemented at the national level. For many years CITES has been among the conservation agreements with the largest membership, with now 180 Parties. Italy joined CITES in 1979. The International trade of *Acipenser naccarii* is subjected to restrictions (CITES II, since 01/04/1998) (www.cites.org).

1.6.3.5 IUCN and the Red List of Threatened Species

The International Union for Conservation of Nature (IUCN) is a governmental and non-governmental organisation working in the field of nature conservation and sustainable use of natural resources. It was established on October 5th, 1948, in Fontainebleau, France. In the past, it has been called the International Union for Protection of Nature (1948-1956) and the World Conservation Union (1990 - 2008).

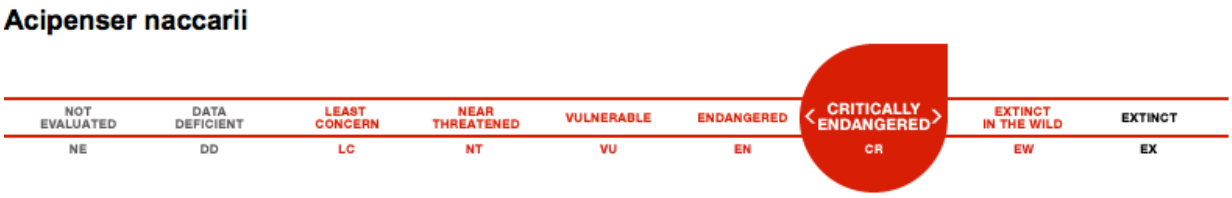
IUCN is involved in data-analysis, research, field projects, advocacy, lobbying and education. Initially its operations were almost exclusively grounded in conservation ecology. Over the past decades, the organisation has widened its scope and now incorporates aspects such as gender equality, poverty alleviation and sustainable business in its activities. IUCN has observer and consultative status at the United Nations, and plays a role in the implementation of several international conventions on nature conservation and biodiversity. It is best known for compiling and publishing the IUCN Red List of Threatened Species, which assesses the conservation status of species worldwide, that indicates whether the species still exists and how likely is to become extinct in the near future.

Species are classified by the IUCN Red List into nine groups set through criteria such as rate of decline, population size, area of geographic distribution, and degree of population and distribution fragmentation. Also included are species that have gone extinct since 500 AD. When discussing the IUCN Red List, the official term "threatened" is a grouping of three categories: critically endangered, endangered and vulnerable (www.iucnredlist.org).

The full list of categories down below:

- *Extinct* (EX) – No known individuals remaining.
- *Extinct in wild life* (EW) – Known only to survive in captivity, or as a naturalized population outside its historic range.
- *Critically endangered* (CR) – Extremely high risk of extinction in the wild.
- *Endangered* (EN) – High risk of extinction in the wild.
- *Vulnerable* (VU) – High risk of endangerment in the wild.
- *Near threatened* (NT) – Likely to become endangered in the near future.

- *Least concern* (LC) – Lowest risk. Does not qualify for a higher risk category. Widespread and abundant taxa are included in this category.
- *Data deficient* (DD) – Not enough data to make an assessment of its risk of extinction.
- *Not evaluated* (NE) – Has not yet been evaluated against the criteria.



Acipenser naccarii was considered in 1996 vulnerable (VU), but in 2009 was evaluated as critically endangered (CR) due to the fast decline of the species, reaching 80% of the population in the last three generations (60 years). The IUCN has also established a group of 40 experts on sturgeons, the SSG- Sturgeon Specialist Group that contributes to the conservation and sustainable use of sturgeon with members in more than ten major sturgeon range states. The SSG works in collaboration with various components of IUCN, such as the Species Programme and TRAFFIC (a joint programme of IUCN and WWF) (www.iucnredlist.org).

1.6.4 National protection measures

1.6.4.1 Protection measures before Habitats Directive

In Italy, the acknowledgment of the Habitats Directive occurred with a D.P.R on 8th September 1997 n.357. Previously to this date, the Adriatic sturgeon was protected by D.P.R October 2nd 1968 n.1639, regulation for the execution of the law n.963, July 14th 1965, on the regulation of sea fishing that established the minimum size of capture to 60 cm (Modified D.P.R. June 9th 1976, n. 1057- D.M. August 4th 1982-D.M. April 21st 1983- D.P.R September 22nd 1978, n.651- D.P.R October 10th 1977, n.920- D.P.R March 18th 1983, n.219).

Now in Italy (DLgs July 7th 2011, n. 121), the capture of *Acipenser naccarii* is prohibited, both in sea and rivers. In case of accidental capture of a specimen, it is required to free him in the water and to report its accidental capture to the fishery offices of the Province.

1.6.4.2 Natura 2000 network

Natura 2000 is the centrepiece of EU nature and biodiversity policy. It is an EU wide network of nature protection areas established under the Habitats Directive. The aim of the network is to assure the long-term survival of Europe's most valuable and threatened species and habitats. It is divided in Special Areas of Conservation (SAC) designated by Member States under the Habitats Directive, and also incorporates Special Protection Areas (SPAs) which were designate under the 1979 Birds Directive. The establishment of this network of protected areas also fulfils a Community obligation under the UN Convention on Biological Diversity. Natura 2000 is not a system of strict nature reserves where all human activities are excluded. Whereas the network will certainly include nature reserves most of the land is likely to continue to be privately owned and the emphasis will be on ensuring that future management is sustainable, both ecologically and economically. Natura 2000 applies to Birds Sites and to Habitats Sites, which are divided into biogeographical regions. It also applies to the marine environment.

In Italy the identification of SPAs is up to the Regions and autonomous Provinces, which transmit data to the Ministry of the Environment. The Ministry, after verification of the completeness and consistency of information collected, transmits the data to the European Commission. SPAs means designated by the date of submission to the Commission and an updated list of SPAs is published on the website of the Ministry, "List of SPAs" (D.M. 8th August 2014) (www.minambiente.it).

1.6.4.3 BE-NATUR - Better management and implementation of Natura 2000 sites

The Habitats and Birds Directives, are part of a solid legal basis for EU nature conservation, and provides the foundation for the preservation of the natural heritage of the European Union. It is known that several countries show different levels of

application of the Guidelines dictated by the Directive, as well as showing strong gap in the management of Natura 2000 sites. BE-NATUR therefore aims to improve the management and implementation of such sites, with particular attention to wetlands (rivers, lakes, coastal areas).

Aims and goals of the project are:

- Definition of a transnational strategy and common tools for better management and implementation of Natura 2000 sites: 1) definition of Transnational Action Plans for habitat and species common to the partner areas (prescribed by the Directives), and implementation of procedures for wide adoption national; 2) definition of a methodology for joint monitoring of results; 3) definition of common guidelines for better management and implementation of Natura 2000 sites;
- Update task for experts for the application of the tools mentioned above;
- Implementation of the strategy through transnational common interventions: 1) Restoration and conservation of habitat (provided in Annex I to Dir. 92/43 / EEC); 2) Reintroduction and conservation of plant and animal species (provided in Annex II Dir. 92/43/ EEC and Annex I Dir. 79/409 / EEC); 3) definition of management plans;
- Actions of dissemination and disclosure towards decision makers and managers of Natura 2000 sites;
- Awareness and environmental education towards schools and families (wwww.be-natur.it).

  In implementing the Plan, the Province of Ravenna has conducted a pilot project for the restocking and reintroduction of the Adriatic sturgeon in a semi-natural environment within the Emilia-Romagna Region, in collaboration with the University of Bologna and Aquarium of Cattolica.

1.6.4.4 LIFE Programme

The LIFE programme is the EU's funding instrument for the environment and climate action. The general objective of LIFE is to contribute to the implementation, updating and development of EU environmental and climate policy and legislation by co-financing projects with European added

value. The programme began in 1992 and to date there have been four complete phases of the programme (LIFE I: 1992-1995, LIFE II: 1996-1999, LIFE III: 2000-2006 and LIFE+: 2007-2013). During the second phase of the programme it has been developed a particular project named LIFE-Nature, set out specifically to contribute to the implementation of the Birds and Habitats Directives, in particular the Natura 2000 network, which promotes the conservation of natural habitats and the habitats of wild fauna and flora while taking into account the economic, social and cultural requirements and specific regional and local characteristics of each State Member. Projects had to target Special Protection Areas or Sites of EU Importance and the species listed in the directives. Projects were chosen purely on their quality and potential conservation impact and not according to national quotas, which ensured that only the very best projects were funded every year.

For what concerns the Adriatic sturgeon it has been developed a special programme, LIFE project 04NAT/IT/000126 “Conservation and Breeding of Italian Cobice Endemic Sturgeon”. For the project “LIFE COBICE” eleven provincial and regional administrations have worked to fulfil the common aim of the conservation of the species, throughout co-financing of the regions of Veneto, Lombardy and Emilia-Romagna and the European Commission. The aim of the programme is to establish a long-term conservation strategy of the *Acipenser naccarii* throughout its Italian area of distribution (www.ec.europa.eu).

1.6.4.5 Action Plan – Lombardy

The conclusion of the programme LIFE COBICE – LIFE04NAT/IT/000126, that took place between 2004 and 2007, was the creation of its *Action Plan* for the species *Acipenser naccarii,* that has been approved by the Lombardy Region with a D.P.R December 21 2007, n.8/6308. The *Action Plan* of the Adriatic sturgeon is an official document that provides guidelines for pursuing all commitments attributed to the different actors of the project.

The main actions are:

- to pursue the monitoring plan to be implemented throughout the network of detection already activated;

- interventions for facilitating the ascent of the sturgeon where dams represents an obstacle for their migration;
- verification of the impact of the hydroelectric plant of Maleo;
- to suggest an update of the standard forms of Natura 2000 sites, along with the Lombardy Region;
- the definition of agreements between institutions to promote actions to improve habitats and safeguard the species;
- to develop strategies to contrast illegal fishing.

The Province of Cremona, partner of the conservation programme for the Adriatic sturgeon, has continued to pursue the aims of the Action Plan during 2010/2011 (www.server.ambiente.regione.lombardia.it).

2. AIM OF THE WORK

The study of this thesis aims to characterize artificial restocking population of Adriatic sturgeon (*Acipenser naccarii,* Bonaparte 1836), with both genetic and physiological analysis in order to establish an efficient restocking programme for future reproduction.

The research is structured on two levels:

- First genetically, by analysing 9 microsatellite loci. This gives information relatively about parent allocation and kinship between individuals that were sampled for this study. Also to predict which reproduction events are the most optimal in terms of incrementing genetic diversity, by the estimation of Multi-locus pairwise band sharing coefficients;
- Second physiologically, by measuring testosterone (T) concentration levels in each individual sampled for sexing, without sacrificing the lives of these animals with the use of an invasive examination of the gonads.

The combination of the two analyses gives the possibility to obtain an overall picture of the population sampled: parent allocations, kinship and sex allocations. This research is important in order to indicate the main broodstock participating in reproduction events and future optimal potential participants, with the use of MAS (marker-assisted-selection), in order to ensure a valid restocking programme.

3. MATERIALS AND METHODS

3.1 Blood sampling

Eighteen specimens of *Acipenser naccarii* were sampled for this study, out of an estimated total amount of 400 animals alive in all Europe. In terms of percentage the 4.5% of the living population is hosted at the breeding farm of the Province of Milan. The animals live in Orzinuovi in a semi-natural environment. They are bred in different artificial tanks, based on the stage of growth, till they reach a certain average size of maturity (>20kg) and released in a common semi-natural pond.

The identification of each single animal was performed by the use of an electro-tagging detection device that gives us information about the microchip ID number (previously implanted), sex of the specimen and the maturity stage (Fig.13). Out of the 18 specimen sampled, 10 were adults and the remaining 8, were represented by offspring specimens, listed in Tab.1. For genetic and physiological analysis, blood samples were collected with syringes from the caudal vasculature (Fig.14). Blood sampling is a very delicate procedure, it is important to minimize stress factors due to the catch of each animal with nets, and during blood collection.

Fig.13. Tag reading.

Fig.14. Blood sampling.

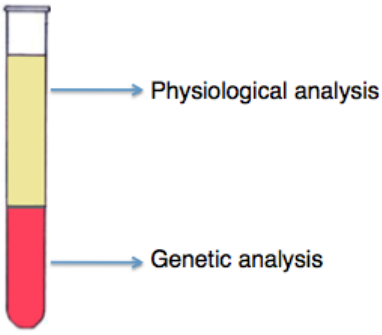


Fig.15 Graphic representation of blood sample, after centrifugation.

A portable lab centrifuge was used *in loco* to separate plasma from particulate suspension, used further for physiological analysis, provided from each blood sampling. The blood samples were collected

in 2 mL centrifuge tubes, each labelled with ID number of the specimen of origin. Both plasma and blood samples were stored at -20°C for further physiological and genetic analysis.

Tab.1 Characterization of population samples.

N.Specimen	Sex	Maturity	Lenght (cm)	Weight (Kg)
1	F	ADULT	145	28
2	?	OFFSPRING	120	14
3	M	ADULT	144	28
4	?	OFFSPRING	125	14
5	?	OFFSPRING	130	23
6	?	OFFSPRING	135	23
7	M	ADULT	140	20
8	M	ADULT	129	20
9	M	ADULT	146	27
10	?	OFFSPRING	130	20
11	?	OFFSPRING	135	25
12	M	ADULT	117	14
13	M	ADULT	130	20
14	?	OFFSPRING	138	22
15	?	OFFSPRING	139	22
16	F	ADULT	120	14
17	M	ADULT	138	21
18	M*	ADULT	185	43

? = sex not assigned, M*= presumed male.

In the studied population there were two peculiar animals: individual N.1 (adult female) and N.18 (adult, presumed male). The female, known as “Albina” (Fig.15) due to her pale livery (but in fact she is not genetically albino) is thought to be the mother of most offspring and the peer or sister of the other adult specimen. The presumed male, known as the “Monster” (Fig.16) due to its length (almost 2 m), was captured in the wild and its origin is in doubt whether it comes from aquaculture and was released during the restocking activities that have taken place since 1992 (Ludwig *et al.,* 2003). At the time of the sampling, all the offspring had not reached complete maturity, thus the sex allocation was still uncertain.

Fig.16 Specimen N.1 (Female “Albina”).

Fig.17 Specimen N.18 (Male “Monster”).

3.2 Genetic analysis

3.2.1 Microsatellite loci selection and laboratory procedures

Genomic DNA was extracted from blood samples (not heparinised) starting from 25 μL of blood, plus175 μL of PBS, using the DNA easy Blood and Tissue Extraction kit (Qiagen) stored at -20°C. After checking the good quality of the extraction throughout electrophoresis analysis on agarose gel 1.8%, follows quantification of the DNA extraction with the use of Qubit fluorometer (Invitrogen).

DNA analysis was conducted on 9 polymorphic microsatellite *loci* listed in Tab.2, especially selected for this study after an accurate research in literature.

The 9 microsatellite loci were PCR amplified, from genomic DNA 50 ng in 20 μL reaction containing: Buffer 1x (4 μL); $MgCl_2$ in different concentration obtained to optimize a successful amplification: 1.5 mM (1.2μL) for *loci* An16, AnacB11, AnacA6 and Spl120, 2 mM (1.6μL) for *loci* LS54 and AoxD234, 1.8 mM (1.4μL) for *loci* Anac B7, AnacE4, Anac B10; dNTPs 0.8 mM (1,6 μL) for all except for *loci* LS54 and AoxD234 0,25 mM (0,5 μL); for each primer (P_F and P_R) 5 μM (0.5 μL) except for *locus* An16 with 4μM (0.4 μL); Taq-polymerase 1 U/μL. Forward primers were fluorescently labelled with NED (yellow) for *loci* AnacA6, AnacE4 and Spl120; 6-FAM (blu) for *loci* AnacB7, AnacB11 and AoxD234; HEX (green) for *loci* AnacB10, An16 and LS54 (Invitrogen and Applera).

Tab.2 Microsatellites *loci* used for this study of *Acipenser naccarii.*

Locus	Reference	GenMap Code	Primer Sequence 5'-3' (P_F, P_R)	MOTIF	Ta
AnacA6	Forlani *et al.* (2008)	EF576943	GCTAGGTGGCTCAGCAGG	(CA)15	60
			GTTGCTTCCTGCATTCCAAGC		
AnacB7	Forlani et al. (2008)	EF576945	GGAGTCCAGGATCCGTC	(GA)23	57
			CCTTCCAGCATGAGTAGC		
AnacB10	Forlani *et al.* (2008)	EF576946	CTAGGAAGCTGTCAAGTTGC	(GTT)17	62
			GGCTGCCGAACCATGTGAC		
AnacE4	Forlani *et al.* (2008)	EF576950	TCAGCTACAGGGTTCTGGG	(CA)20	55
			GTTGTTACTCATTGGAAGTC		
AnacB11	Forlani *et al.* (2008)	EF576947	GCAGCAGAATTCAGAACATG	(CA)9AA(CA)10	54
			TGATGGAACACAAGACAGTG		
An16	Zane *et al.* (2002)	AY144617	TTAACCACTGGACCACACAGCA	(ATCT)24	62
			TCCCACCATGCACCACACTAGA		
Spl120	McQuown *et al.* (2000)	AF276189	ATTCCATGAGCAACACCACA	(TATC)15	57
			TGATGGTCTGATGAGATCGG		
AoxD234	Henderson-Arzapalo (2002)	AY093645	AACTGGCTTTGTGATTGATCC	(TAGA)17	56
			TGAAGCAAAGGGTATTATTTGAG		
LS54	May *et al.* (1997)	U72735	TCTKTCAAGGTATACTTTAG	(GATA)6	57
			GCAGACAAAATAGCATTCAG		

MOTIF (nucleotide sequence), Ta (annealing temperature).

PCR reactions were performed in a Personal T-Gradient thermocycler (Biometra). The temperature profile consisted of an initial denaturation step at 94°C for 4 min, followed by 32 cycles of denaturation, for all locus except AnacA6, B11, Spl120, LS54 and AoxD234 with 35 cycles, at 94° C for 30 s, annealing at the optimized Ta (see Tab.2.) for 30 s, elongation at 72°C for 10 min.

The amplicons were separated by capillary electrophoresis by a commercial provider (BMR Genomics), using the ABI3100 DNA analyser. The electropherogram files were imported into the software Peak Scanner 1.0 (Applied Biosystems), and then the microsatellite alleles were sized and scored to infer the individual genotypes.

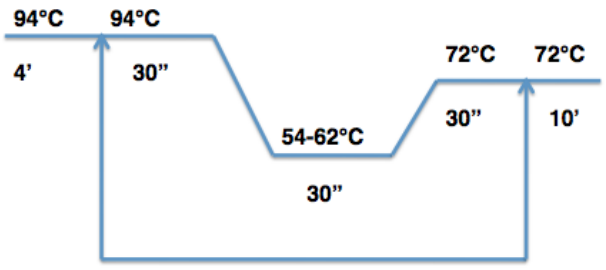


Fig.18 PCR profile cycle.

3.2.2 Genotyping

Alleles classes were identified by Peak Scanner 1.0 by assigning a discrete and integer value to the alleles detected. It is necessary to take in consideration that difficulties can occur due to the tetraploidy condition of *Acipenser naccarii*; up to four alleles per locus can be present and six combinations can be formed in equal proportions in the case of tetrasomic inheritance. Only in case of mono- and quadriallelic classes there is an association between phenotype and genotype, otherwise hidden alleles will be presented in DNA-profiling (De Silva *et al.,* 2005).

3.2.3 Genetic variability

Given the tetraploid condition of the Adriatic sturgeon and the consequence impossibility to estimate the allelic frequencies, the variability of the loci used was assessed by estimating the Intralocus Band Sharing (IBS). This parameter estimates, for each single locus, the number of allelic classes shared by all samples individuals, the number of alleles per locus and the maximum number of bands per individuals (Forlani *et al.,* 2008).

3.2.4 Parental allocation

For Parents Allocations (PA), specific software was used for tetraploid organisms-wHDP. The wHDP produces PA, using co-dominant or dominant markers, by exclusion method and in the case of multiple allocations it is used a likelihood (**L**) approach to select the most probable parents-pair (Galli *et al.*, 2011). The exclusion method, based on Mendelian mode of inheritance: the rejection of one parent-offspring hypothesis is based on incompatibility of DNA profiles. This method is good when the candidate parents are not numerous and the genetic markers are highly polymorphic.

In tetraploids, given the impossibility to measure correctly the allele frequencies, **L** is computed using an equation derived from gamete multivariate hypergeometric distribution:

$$L_n = \begin{pmatrix} s \\ r \end{pmatrix}_C \begin{pmatrix} n \\ r \end{pmatrix}^{-1} \cdot \begin{pmatrix} s \\ r \end{pmatrix}_D \begin{pmatrix} n \\ r \end{pmatrix}^{-1}$$

n= number of alleles per locus (*n*=4);

r = number of alleles per locus in gamete (*r*=2);

s = number of alleles of parents (C or D) present in offspring.

The total likelihood is computed:

$$\mathbf{L} = {}^{n}\Pi\, L_n$$

In the case of multiple allocations the difference between the first two ranked L values (ΔL) is computed and PA with ΔL>0 is accepted and the multiple allocations can be considered as "resolved". While with ΔL=0 the multiple allocation is defined ambiguous (Galli *et al.*, 2011).

3.2.5 Choice of selective crosses

In order to increase genetic variability of future offsprings, it is necessary to mate individuals which are not related to each other and which are the most genetically differentiated. For this purpose, it is necessary to use the Multilocus Pairwise Band Sharing coefficient (MPBS) calculated according to Zhu *et al*., 1996:

$MPBS = 2N_{AB}\,(N_A + N_B)^{-1}$;

N_{AB}= number of bands present in both subjects;

N_A= total number of bands present in subject A;

N_B= total number of bands present in subject B.

In order to automate and speed up the evaluation of MPBS a phyton scripts has been developed specifically for this work. This programme is capable to analyse a csv file containing the acquired data (Locus, Alleles, Gender) and provides as output a report that lists, for each Female/Male couple, the computed band sharing parameters with detail of matching locus. The script can be freely downloaded at: https://github.com/beardbig/bandsharing, https://github.com/beardbig/bandsharing/archive/master.zip.

3.3 Physiological analysis

3.3.1 Radioimmunoassay (RIA)

Radioimmunoassay (RIA) is an *in vitro* assay that measures the presence of an antigen with very high sensitivity and any biological substance for which a specific antibody exists can be measured. The target antigen is labelled radioactively and bound to its specific antibodies (a limited and known amount of the specific antibody has to be added) (Patrono and Peskar, 1987). A sample, for this study a blood serum, is then added in order to initiate a competitive reaction of the labelled antigens from the preparation, and the unlabelled antigens from the serum-sample, with the specific antibodies. The competition for the antibodies will release a certain amount of labelled antigen. This amount is proportional to the ratio of labelled to unlabelled antigen. A binding curve can then be generated which allows the amount of antigen in the sample serum to be derived. That means that the more increase the concentration of unlabelled antigen, the more of it binds to the antibody, displacing the labelled variant. The bound antigens are then separated from the unbound ones, and the radioactivity of the free antigens remaining in the supernatant is measured (Patrono and Peskar, 1987).

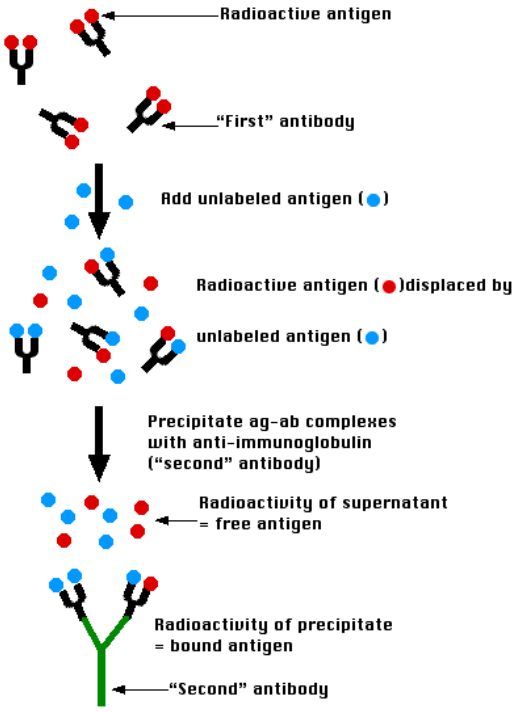


Fig.19. Schematic representation of RIA (www.users.rcn.com).

3.3.2 Laboratory procedures : radioimmunoassay of Testosterone (T)

Testosterone concentration was determined using a validated RIA (Gaiani *et al.,* 1984), partly modified. The hormone was extracted from plasma samples (0.2 mL) with 5 ml of diethyl ether by stirring on "rotor" for 30 minutes and centrifugation at 2000g for 4 min. At a rate of 0.1 mL, was added tritiated testosterone (30 pg / tube) and 0.1 mL of a solution of 1: 50,000 of antibody anti-testosterone.

The dried extracts were dissolved in 1 mL of an RIA-phosphate buffer (Na_2HPO4

74.26 mmol/L, EDTA Na 12.49 mmol/L, NaN_3 7.69 mmol/L) containing 0.1% bovine serum albumin, pH 7.5, and were shaken for 10 min. The sample (0.1 mL), 1,2,6,7-^{3}H testosterone (T) (0.1 mL, 30 pg/tube) and rabbit anti-testosterone serum (0.1 mL, 1:50,000) were incubated overnight at 4°C; 1 mL of charcoal-dextran solution (charcoal 0.25%, dextran 0.02% in phosphate buffer) was then added to the tubes. After 15 min at 4°C, the tubes were centrifuged for 15 min at 3000 x *g*, the supernatant was decanted and radioactivity was immediately measured using a β scintillation counter (Packard C1600, PerkinElmer, USA). In parallel to the ether extracts of plasma, the assay was conducted on known quantities of the hormone, in order to set up a curve reading. The average recovery, evaluated in preliminary tests, was 80%.

The cross reactions of various steroids with the rabbit anti-testosterone serum were as follows: testosterone 100%, DHT 25.4%, dione 0.43%, cortisol <0.001%, progesterone <0.001%. Verifying that serial dilutions were parallel to standard curves validated testosterone level determined by RIA.

4. RESULTS

4.1 Genetic Analysis

4.1.1 Genetic variability

The overall genetic variability was defined by several parameters: observed allelic range, number of alleles per locus, the maximum number of alleles per locus/individual and the intralocus band sharing parameter expressed both in value and percentage (Tab.5, Fig.21). Given the tetraploid condition of the Adriatic sturgeon and the consequence impossibility to estimate the allelic frequencies, the variability of the loci was assessed by estimating the number of alleles per locus, the maximum number of bands per individuals and the average intra-locus band sharing among pairwise individuals (Forlani et al., 2008).

Tab.3 Intralocus Band Sharing Parameter (IBSP).

Locus	Observed allele range (bp)	No.of alleles	Max n° of alleles per individual (N=18)	Intra-locus band sharing parameter	Mean percentage of shared bands*
AnacA6	291-305	7	4	0.37	37%
AnacB7	151-173	7	4	0.40	40%
AnacB10	207-258	11	4	0.40	40%
AnacE4	333-355	9	4	0.31	31%
AnacB11	131-163	10	4	0.33	33%
An16	176-204	6	3	0.29	29%
Spl120	264-304	6	4	0.51	51%
AnoxD235	214-274	9	4	0.46	46%
LS54	136-190	7	4	0.43	43%

* = Between individuals, within loci.

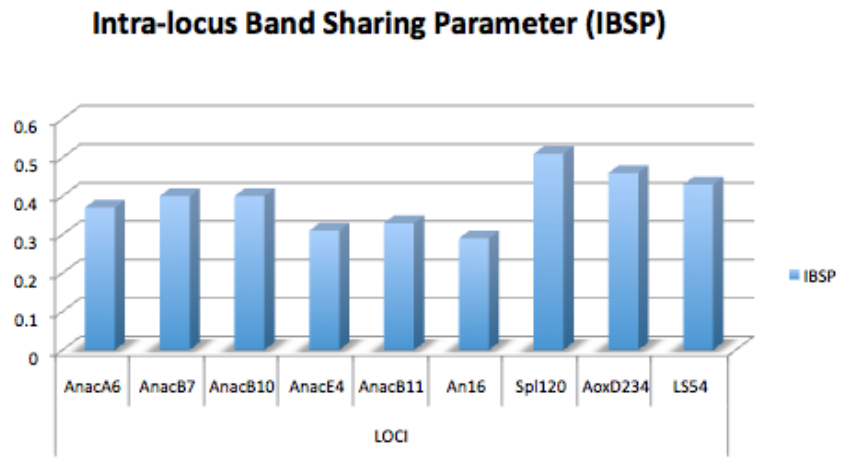


Fig.20 Intra-locus Band Sharing parameter for each of the 9 loci analysed.

4.1.2 Parental Allocations and Kinship

The results of Parental Allocations are listed in Tab.3. The computer procedure was able to detect all the correct pairs of parents.

Complete allocations 4 out of 8:

I) Mother N.1 × Father N.9 = Offsrping N.6;

II) Mother N.16 × Father N.17 = Offsrping N.11;

III) Mother N.16 × Father N.9= Offsrping N.14;

IV) Mother N.16 × Father N.9= Offsrping N.15.

Incomplete allocations 4 out of 8, of which:

- 1 resolved for (ΔL= 0,085):

V) Mother N.16 × Father N.3= Offsrping N.4.

- 3 ambiguous (ΔL= 0), with only one parent assigned:

VI) Mother N.16 × Father ?= Offsrping N.2;

VII) Mother N.16 × Father ?= Offsrping N.5;

VIII) Mother ? × Father 9= Offsrping N.10.

The most likely Kinship, both vertical (parents-offspring) and horizontal (siblings) is possible to observe in Fig.19, with in evidence full-sibs (FS) and half-sibs (HS). From the data obtained it was possible to determinate which adults participated optimistically

in reproduction, and which have probably or did not participate. It is assumable that 5 adults presumably participated in reproduction (N.1F, 3M, 9M, 16F and 17M), one probably participated (18M*) and 4 did not participate (N.7M, 8M, 12M and 13M).

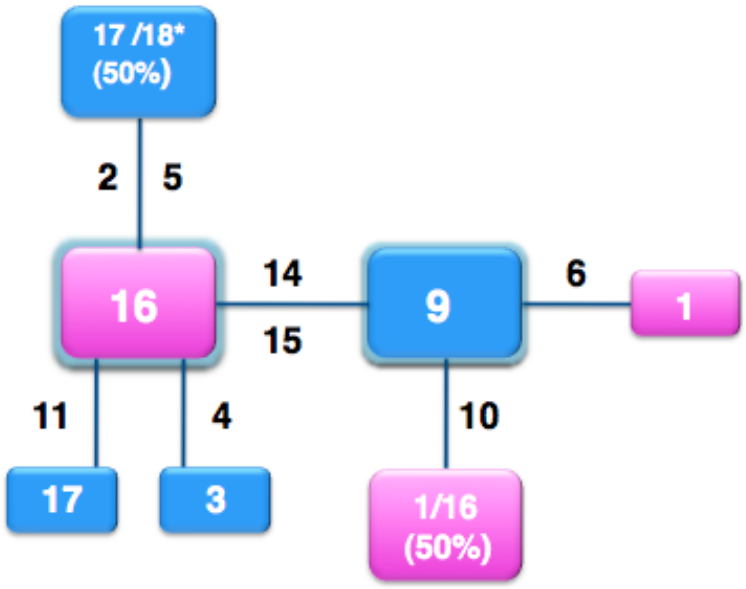


Fig.21 Kinship (FS and HS) between individuals of *Acipenser naccarii*. In the squares: the Id number and the sex (pink female, blue male) of adult individuals. Along the lines: the ID number of the offspring individuals. 18* = assumed male.

The adult number 16 F and 9 M, participated at the most number of reproduction.

Individuals with both parents in common (Full-sibs):

- Offspring N.14 and 15, as the result of reproduction event between adults N. 16 F and 9 M;
- Offspring N. 5 and 2, as the result of reproduction event between adults N.16 F and 17 M or 18M* (50% of probability).

Individuals with only one parent in common (Half-sibs):

- Offspring N.2, 4, 5, 11, 14 and 15 as the result of reproduction event between adults N. 16 F and with at least 3 males or more (N≥3);
- Offspring N.6, 10, 14 and15, as the result of reproduction event between adults N.9 M and 2 or more females (N≥2).

4.1.3 Choice of selective crosses)

After the first discrimination throughout the paternity test, it was possible to decided which marker assisted selection (MAS) were preferable to choose from, based on genetic data obtained by applying the MPBS described previously.

Low values of MPBS (< 0.25), are assumed to be generated between unrelated individuals (Jeffrey *et al*., 1985; Dunnington *et al*., 1994; Zhu *et al*., 1995) and that share the least number of bands. Hence, a low BSC is considered a positive result, meaning that the individuals pairwise compared own high genetic diversity and can compete to amplify the variability of genetic pool of future offspring. For this reason, it is important to focalize on the possible reproduction events that show the value of band sharing with the lowest rate.

In Tab.4 and Tab.5, are listed relative MPBS for each potential MAS (marker-assisted-selections) decided to obtain. In order to estimate each MAS, it is necessary to know the sex of the offspring that are involved; this is determined by physiological analysis, which results are showed in section 5.2.

Tab.4 Potential MAS between Offspring.

ID N. Female	ID N. Male	MPBS
6	4	0.44444
6	5	0.44444
10	4	0.33333
10	5	0.44444

For the choice of possible MAS between Offspring, half-sibs and full-sibs were excluded. The selection of the males N.4 and N.5, were based on the fact that at the time of sampling, the individuals were found fluent. For the calculation of MPBS between offspring 6F × 4M and 10F × 4M, the loci Spl120, AoxD234 and LS54 were ignored because resulted incomplete for the offspring N.4, despite repeated amplifications. As shown in the table, the lowest BSC is between 10F × 4M (MPBS= 0.33333).

Tab.5 Potential MAS between female Offspring & Adult males.

ID N.Female	ID N.Male	MPBS
6	18	0.23255
10	18	0.23809
15	18	0.31578
2	9	0.34783
11	9	0.44444
2	8	0.45455
2	13	0.45833
11	18	0.47619
11	12	0.50000
15	12	0.50000

Inbreeding with parents was excluded *a priori* from the analysis. Full list of MAS between offspring and adults, are listed in Appendix A. To show an example, in Tab.5 are listed 10 MAS between female offspring and adult males based on MPBS; listed in an increasing order from the most optimal MPBS (low value) to the least optimal MPBS (high value). The lowest value of MPBS is represented by reproduction event between individuals N.6F × N.18M (MPBS = 0.23255), also between N.10F × N.18M (MPBS = 0.23809). Locus An 16 was excluded from the calculation of the MPBS for N.2F × N.9M. Also locus LS54 was excluded for N.6F × N.18M and N.10F × N.18M. Full list of MAS between offspring and adults, are listed in Appendix A.

4.2 Physiological analysis

4.2.1. Testosterone (T) concentration levels (ng/mL) in plasma samples

As shown in Tab.6 and Tab.7, it is possible to observe the levels of Testosterone (T) (ng/mL) shown in each animal sampled on April 3rd 2014.

Tab.6 T levels in adult

ID N.Adult	T level(ng/mL)
1	81.24
3	394.58
7	315.02
8	338.56
9	283.78
12	291.02
13	351.38
16	190.05
17	348.67
18	191.45

Tab.7 T levels in offspring

ID N.Offspring	T level(ng/mL)
2	7.2
4	320.10
5	571.35
6	9.48
10	3.49
11	2.17
14	377.96
15	4.75

Two compared scatter plots showing concentration of testosterone (T) levels (ng/ml) in plasma samples, both for adult specimen and offspring, are shown in Fig. 22.

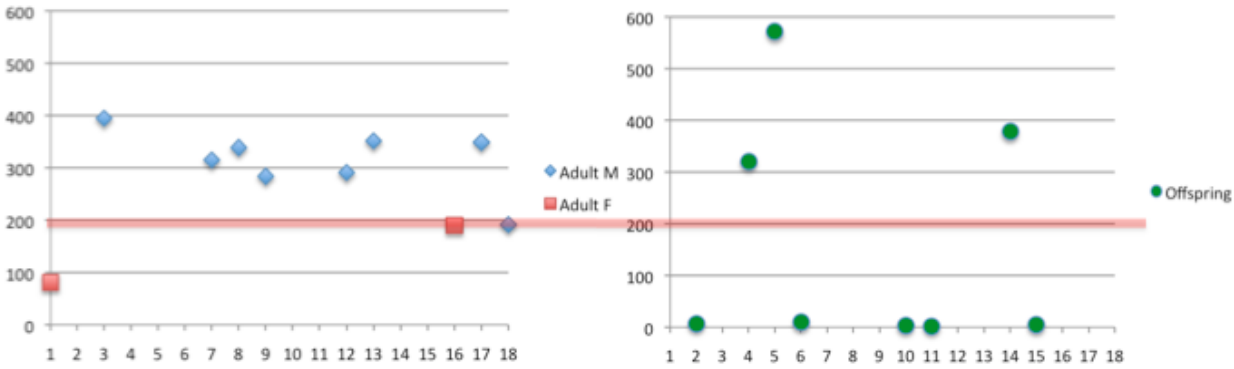


Fig. 22 Scatter plots of T concentration levels in plasma samples respectively for Adult and Offspring specimen. On the x-axis of the scatter plots are indicated the ID number of the individuals; on the y-axis are shown the concentration levels of T expressed in ng/mL.

The red line indicates the limit (range between 195 and 205 ng/mL) over which are present male individuals (blue rhombus) and under the red line, female individuals (red squares). Two adult animals with opposite sex, N.16F (T= 190.05) and N.18M (T= 191.45) show similar concentration of testosterone and are positioned close to the limit indicated by the red line.

5. DISCUSSION AND CONCLUSIONS

5.1 Genetic Analysis

The parental allocation in aquaculture allows the most probable association between progeny and broodstock. Fingerprinting is required for this kind of analysis and microsatellites are the markers mainly used for this purpose. The combination of 9 highly polymorphic nuclear loci, used for this study, resulted efficient in order to assign the F1 progeny to the most likely parental pairs. Form the results obtained by specific software it was possible to assign direct complete parental allocation (both father and mother) to the 50% of the offspring. The rest 50% were assigned incomplete allocations, meaning that was assigned only one of the two parents. Only one incomplete allocation was possible to resolve with a value of $\Delta L > 0$, a likelihood approach used to identify the correct allocation.

During the calculation procedure it was necessary to take in consideration two individuals that showed partial genotypes:

- Adult N.3, loci excluded: An16, Spl120, AoxD234 and LS54;
- Offspring N.4, loci excluded: Spl120, AoxD234 and LS54.

It was necessary to eliminate 2 loci (An16 and LS54) that gave problems during the running of the programme wHDP (Galli et al, 2011), because the software works only in case it is possible to assign all 9 loci at the same time or else it is necessary to run one locus at a time

It is necessary to consider that parental allocation in tetraploids, like *Acipenser naccarii,* shows more difficulties than diploids because up to four alleles per locus can be present and six gamete combinations be formed in equal portions.

- 4 alleles: *abcd;*
- 6 combinations in case of tetrasomic inheritance: *ab, ac, ad, bc, bd, cd.*

The result of all combination gives four phenotypic classes: mono-, bi-, tri- and quadriallelic. Excluding the presence of null-alleles (allele which is not amplified by a specific set of primers), only in the case of the mono- and quadriallelic classes it is possible to associate between phenotype and genotype, otherwise hidden alleles are present: one microsatellite band represents the same allele repeated more times, and in this case different genotypes will be hidden. Thus it is impossible to estimate allele

frequencies. For diploid organisms the hidden alleles represent the homozogosity and the DNA profiling is coded with band value repeated twice. For *Acipenser naccarii*, tetraploid species, it is impossible to detect partial homozigosity, and in this way the information present in false null-alleles is lost.

From the data obtained it was possible to determinate which adults participated most likely in reproduction and/or did not participate. It is assumable that 5 adults participated in reproduction (N.1F, 3M, 9M, 16F and 17M), one probably participated (18M) and 4 did not participate (N.7M, 8M, 12M and 13M).

The reason why these 4 male adults did not contribute in reproduction could be clarified with a future test on the quality of the sperm. For sure, no human activities did occur in the area that could represent an obstacle to reproduction for these animals. No fishing or any kind of intervention is allowed in the area, except for authorized and qualified staff. Furthermore, the semi-natural environment is highly controlled in order to maintain all the optimal physiological ranges for growth and reproduction, and no cannibalism is expected to occur for these animals. Males have always been randomly chosen among the available broodstock. This might explain the possibility that some males have never been used for reproduction. With reference to male 18 a sperm quality test should be eventually carried out.

After the parental allocation analysis it was possible to investigate which selective cross can be applied based on the calculation of the multi-locus pairwise band sharing (MPBS), in accordance with both vertical and horizontal kinship obtained, as shown in Fig. 19.

Low MPBS value is considered an optimistic result, meaning that the individuals that participate in the reproduction event are presumable not related, possessing high genetic diversity and can compete to amplify the genetic pool of the population. Low values of MPBS (< 0.25) are generated between individuals that share the least number of bands during genetic analysis and are assumed to be two individuals that seem truly unrelated, which is reasonable based on the comparison with MPBS obtained in other organisms like chicken, turkey and human population (Jeffrey et al., 1985; Dunnington et al., 1994; Zhu et al., 1995). The MPBS calculated for different reproduction combinations that show values < 0.25, under the threshold value, are the ones that consider the individual N.18, as shown in Tab. 5.

The most interesting individual in this study, for its genetic characteristics (wide variability overall loci), is the assumed adult male N.18, which at first was thought to be a wild specimen, for it was accidentally recovered from a wild environment in 2008.

According to the MPBS, that are significantly low in pairwise comparison in which it is involved, we could be optimistic in assuming that it is actually a wild individual and that can effectively compete for future reproductions, independently on the sex that will need to be ascertain with other methods. However, this animal might have not yet reproduced. The causes are still object of study and the physiological analysis could try to explain the causes of such a low percentage of reproduction.

5.2 Physiological analysis

Efficient studies on gonadal development in *Acipenser naccarii,* for a better sex ratio management, it has been carried out by sacrificing a big amount of animals through histopathology (Grandi *et al*., 2008). Physiological analyses based on the measurement of the sexual steroids concentration from plasma samples, were conducted in order to establish the sex of the individuals without compromising their lives and stress levels. A wider application of these analysis could be used to keep on monitoring the concentration levels of sex steroids during time in order to establish when the animal enters the pre-reproductive phase; in fish the androgens 11-KT and T increase gradually as spermatogenesis proceeds, and reach peak levels shortly before or at the beginning of the spawning season (Shulz *et al*., 2011). As a standard procedure, in order to synchronize the reproduction both males and females are hormonally induced with an injection of LHRH, around ten hour before the expected spawning, and then kept in a separated area of the main pond.

From the scatter plots shown in Fig.21, it is possible to compare the levels of testosterone collected from adult individuals that have already a confirmed sex, with the offspring, still to be sex assigned. Focusing on the concentration trend of the offspring, there are 2 groups:

- First group with testosterone at basal levels: individuals N.2, 6, 10, 11 and 15;
- Second group with high testosterone levels: individuals N.4, 5 and 14.

The two animals N.4 and N.5 were found to be fluent at the time of spawning, confirming the fact that, like in most of teleost, males reach maturity before females, thus leading to the assessment of the sex. Also for what concerns the adult specimen, it is possible to detect 2 groups, like the ones for the offspring. Basal levels of T correspond to females N.1 and 16, and high levels of T to male animals. Adult female N.16 shows higher T levels compared to N.1 due to the fact that, from the information

obtained from the farmer, female N.1 did reproduce in 2013. From what expect for this species, females are not going to reproduce for at least 1-2 years. Instead N.16, at the time of sampling (April 2014), produced significant T levels in order to convert it into the production of E2 for the spawning season of June 2014.

5.3 Genetic and physiological characteristics of individuals N.16 and N.18

From the population sampled for this study, two individuals, N.16 and N.18, showed peculiar aspects. Both presented similar T concentration level, respectively 190.05 and 191.45 (ng/mL).

Information regarding female individual N.16:

- From genetic analysis it is possible to see that is the female that has participated at the most number of reproduction events; N.16 is the mother of offspring N.2, 4, 5,10,11,14 and 15.
- From physiological analysis, testosterone (T) level sampled is not considered relevant as it falls out of the critical range, and has actually contributed in reproduction events. So it is possible to eliminate the eventuality of hormone production or regulation deficit.

Information regarding male N.18:

- From genetic analysis it is possible to see, from the kinship shown in Fig.19, that this individual has probably contributed in reproduction between female N.16 (50% probability) and is the hypothetical father of offspring N.2 and 5. It is extremely important to investigate on the actual reproduction activity of this animal because is presumably considered wild specie, confirmed by the genetic data collected (low levels of BSC) and can contribute to future optimal inbreeding events.
- From physiological analysis, the testosterone level detected, for this particular individual, is not to be considered alarming (low levels compared with other male individuals), though it is advisable to carry out further investigations. In addition, from information given from the farmer, the animal showed very small growth after its placement in the restocking farm in Province of Milan, and over went a stressful period after 2-3 years. This condition might have influenced its capability to reproduce on a long term. A long-term stressed condition in *Acipenser naccarii* influence the over production of cortisol

(Marco *et al*., 1999). Inhibitory effects of cortisol have been demonstrated at all levels of the reproductive axis (although not studied in all species): the gonadotropin-releasing hormone (GnRH) producing neurons in the hypothalamus, the pituitary cells producing the gonadotropins I and II, and the gonadal cells producing the sex steroids (Wendelaar Bonga, 2011).

5.4 Future aims

The combination of both genetic and physiological analysis represents a valid instrument for an optimal and complete investigation of the population sampled, and for the management of artificial restocking program of *Acipenser naccarii*.

The genetic characterization of all the breeders present in a restocking farm will allow setting up a long-term program aiming in optimizing the future MAS. Following, by genotyping all of the individuals using microsatellite markers, it is possible to summarize all the information needed between possible reproduction pairs (based on MPBS values). This will facilitate the choice of candidate breeders that are genetically different.

At the present state there is no evidence of natural reproduction for the Adriatic sturgeon in wild environment, which makes this species dependent on captive breeding programs that have to be improved. The application of the protocol used for this study may suggest a better way of administrating current and future stocking programmes for *Acipenser naccarii*.

The ultimate goal would be the re-introduction of this species in its natural habitat. In parallel with genetic and physiological analysis, for achieving an optimal reintroducing plan it is necessary to integrate and monitor information regarding factors affecting the natural habitat, including restoration of spawning sites and establishment of fish passage of dams.

5.5 Conclusions

The peculiar ecology and biology of *Acipenser naccarii* (restricted areal and anadromy) makes this specie particularly vulnerable to fast environmental changes due to increasing anthrophization and unsustainable use of natural resources. The Adriatic sturgeon, in its present status, is extinct in most of its original range of distribution (Forneris *et al.*, 2012). Latest data, about natural spawning, date back to the early 80s. For this reason, the species is now protected by EU laws, which prohibit capture and killing and is subjected to recovery programmes (Porcellotti, 2005). This specie is also included in the “Red list” on the conservation status of the species of the International Union for Conservation of Nature (IUCN) and was considered in 1996 as vulnerable, but in 2009 was evaluated as critically endangered (extremely high risk of extinction in the wild) due to the fast decline of the species, reaching 80% of the population in the last three generations (60 years) (www.iucnredlist.org).

Due to its rapid decline, starting from the ’80 till now, at the present state there is no evidence of natural reproduction for the Adriatic sturgeon in wild environment, which makes this species dependent on captive breeding programs that have to be improved.

The methods applied in the present study have proved to be efficient in the contest of the management of a small population that needs to be carefully managed during all the steps of the breeding process.

The combination of interdisciplinary analysis, used in this study, can lead to optimizing management programs of captive breeding restocking in three steps:

1) Physiological analysis for early detection of the individual sex and its readiness for the treatments leading to the breeding season;
2) Genetic analysis for the choice of the best possible crosses to obtain a genetically optimal stock;
3) MAS (marker-assisted-selection), the execution phase and ultimate goal of the program, in which the animals are specifically selected and bred on the basis of the outcomes of the analysis process.

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APPENDIX A

ID N. Female	ID N.Male	MPBS
6	18	0.23255
10	18	0.23809
15	18	0.31578
2	9	0.34783
11	9	0.44444
2	8	0.45455
2	13	0.45833
11	18	0.47619
11	12	0.50000
15	12	0.50000
10	12	0.51064
11	13	0.51064
6	13	0.52174
10	8	0.52381
2	12	0.53061
6	12	0.55319
11	8	0.55814
15	8	0.56410
15	13	0.60465
10	13	0.60870

Appendix A: List of possible Potential MAS between female Offspring & Adult males.

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