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CYCLE XXIX

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Integrating genetic analyses and morpho-cellular approaches to sustainably conserve the marble trout (*Salmo marmoratus* Cuvier, 1817) population.

Scientific/Disciplinary Sector (SDS) BIO05/BIO07

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Abstract

The 2013 IUCN Red List included the marble trout (*Salmo marmoratus*), already listed in Annex II of EU Habitat Directive (92/43/EEC), in the Critically Endangered (CR) category although in the last decades many project were dedicated to the conservation of the taxon. Since the beginning of the 20th century the brown trout (*Salmo trutta*) has been introduced in the habitat of marble trout. This overlapping of distribution range resulted into hybridization: the native marble trouts are nowadays rare in most rivers and this determines a low number of breeders in the wild and a low production of pure offsprings every year. Regarding this, nowadays is fundamental a genetic carachterization of the breeders to ensure a high level of selection of the individuals that has to be directed to the reproductive career. The aim of this work is to select genetic strains of marble trout in order to breed them in a selected structure finalised in fish species conservation. For this reason, integrating genetic analyses and an innovative approach based on morpho-cellular quali-quantitative evaluation can lead to the accomplishment of a live gene-bank, a hatchery whose aim is to breed higly selected offsprings for restocking purposes. 229 fish were submitted to a strict phenotypic selection based on some peculiar morphological traits, before the transfer in the hatchery, then were tagged with a PIT-TAG to identify them later. The D-loop region and nuclear gene LDH- C1* were amplified to exclude hybrid individuals before next analyses. On a subset of 90 individuals resulting marble/Mediterranean, for the mtDNA and nDNA analyses, and an outgroup of 24 Mediterranean trout (*Salmo cettii*) was run a panel of 15 microsatellites in order to investigate the genetic diversity. The analyses showed a clear difference between individuals from the three different basins therefore, for the artificial fertilization, the three populations were maintained separated. However, from the plot was also evident a genetic pollution in the trouts from two hatcheries and one individual showed even a high percentage of genetic similarity with Mediterranean trouts proving the efficacy of the analyses conducted. Sperm motility and milt concentration were measured in the hatchery during the reproductive season by dark-field microscope and SDM6 photometer. Milt from the individuals that showed higher values of genetic variability has been used for the artificial fertilization and cryopreserved for future breeding. Nine males were sampled periodically in order to monitor the possible milt concentration variation during the reproductive season. In addition an egg fertilization experiment was conducted to test some artificial fertilization product.

Combining molecular tools and innovative techniques can be an important innovation in hatcheries both for commercial and conservation purposes. Being able to select and cryopreserve gametes of marble trout breeders and that carry the higher genetic variability is really important in order to maintain endangered species.

Integrating genetic analyses and morpho-cellular approaches to sustainably conserve the marble trout (*Salmo marmoratus* Cuvier, 1817) population.

Abstract

La Lista Rossa IUCN del 2013 ha classificato la trota marmorata (*Salmo marmoratus*), già presente nell'Allegato II della Direttiva Habitat (92/43/EEC), "a maggior rischio" (CR, Critically Endangered) nonostante numerosi progetti negli ultimi decenni siano stati dedicati alla salvaguradia del taxon. Fin dall'inizio del 20esimo secolo negli habitat della trota marmorata è stata introdotta la trota fario (*Salmo trutta*). Questa sovrapposizione nei range di distribuzione ha avuto come risultato l'ibridazione: le trote marmorate autoctone oggigiorno sono rare, nella maggior parte dei fiumi, determinando un numero basso di riproduttori in natura e una bassa produzione di prole pura ogni anno. A tal riguardo è oggi fondamentale una corretta caratterizzazione genetica dei riproduttori per assicurare un alto livello di selezione degli esemplari da avviare alla carriera riproduttiva. Lo scopo di questo lavoro è di selezionare ceppi genetici di trota marmorata per allevare gli animali in strutture selezionate finalizzate alla conservazione di specie ittiche. A tal proposito, l'integrazione di analisi genetiche e di un approccio innovativo basato su una valutazione quali-quantitativa morfo-cellulare può portare alla creazione di una live gene-bank, ossia di un allevamento il cui scopo è quello di produrre progenie altamente selezionata per scopi di ripopolamento. 229 pesci sono stati sottoposti ad una rigida selezione fenotipica, basata su alcuni tratti morfologici peculiari, prima del trasferimento in allevamento, quindi sono stati taggati con un PIT-TAG per poterli identificare in seguito. La regione della D-loop e il gene nucleare LDH- C1* sono stati amplificati per escludere gli ibridi prima delle analisi seguenti. Per indagare la diversità genetica è stato testato un pannello di 15 loci microsatelliti su un sottoinsieme di 90 individui risultati marmorata/Mediterranea, per le analisi sul mtDNA e sul nDNA, e su un outgroup di 24 trote mediterranee (*Salmo cettii*). Le analisi hanno mostrato una chiara differenza tra gli individui provenienti da tre diversi bacini fluviali e di conseguenza, per le riproduzioni artificiali, le tre popolazioni sono state mantenute separate. Tuttavia, dal grafico ottenuto è stato evidente anche un inquinamento genetico nelle trote provenienti da due allevamenti e un individuo ha mostrato un'alta percentuale di somiglianza genetica con l'outgroup di trote mediterranee, confermando l'efficacia delle analisi condotte. La motilità spermatica e la concentrazione del liquido seminale sono stati misurati nell'allevamento durante la stagione riproduttiva con un microscopio ottico e il fotometro SDM6. Il liquido seminale degli individui che hanno fatto registrare i valori più alti di variabilità genetica è stato utilizzato per le fecondazioni

artificiali e crioconservato per futuri accoppiamenti. Nove maschi sono stati campionati periodicamente per monitorare la possibile variazione nella concentrazione spermatica durante la stagione riproduttiva. In aggiunta è stato condotto un esperimento con la fecondazione di uova per testare alcuni prodotti commerciali.

Combinare strumenti molecolari e tecnologie innovative può costituire un'importante innovazione negli allevamenti sia a scopo commerciale che di conservazione. Essere capaci di selezionare e crioconservare i gameti di riproduttori con alta variabilità genetica di trota marmorata pura è veramente importante per preservare specie in pericolo.

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1. Introduction

The freshwater fish fauna in Italy experienced serious modification because of the big progression that, after the World War II, made both agriculture and industry and because of the numerous anthropic activities connected (Nonnis Marzano *et al.*, 2002). Climate changes, habitat shifts and modifications, pollution, alien species and a bad management in natural resources, led to a sufficiently problematic situation; in fact, most autochthonous taxa are under severe threat or already extinct on a local or national scale (Zerunian, 2002; Nonnis Marzano *et al.*, 2014). In this respect, the recent IUCN Red List review (Rondinini *et al.*, 2013) regarding 49 autochthonous Italian fish species (of which 29 Osteichthyes and Agnatha are inserted in the Habitat Directive) highlighted the seriously compromised status of freshwater fishes population. If we consider both the settled and the diadromous species, in the application of IUCN (International Union for the Conservation of Nature) parameters, in Italy we record 2 extinct species at a regional level (RE), 11 critically endangered (CR), 6 endangered (EN), 3 near threatened (NT), 8 vulnerable (VU), 6 with data deficient (DD) and only 13 least concerned (LC). Italy has an interesting geological history: its particular geographical positioning, surrounded by the sea, divided by the Apennines and separated by the Alps from the rest of the Europe led to the differentiation of many endemic species (Zerunian, 2002). If we consider Italy from an ichtyo-geographical point of view, we can state that this country is a true biodiversity hotspot because almost half of indigenous species are endemisms or sub-endemisms (Bianco, 1996).

Demographically speaking, invasive species and habitat fragmentation, due to water diversions or dams created for hydroelectricity and the collection of water destined to zoo technical purposes or irrigation systems, can severely affect the solidity of the indigenous population in lowland sections of Po valley basins. Moreover, population dynamics of several fish species do not seem reassuring in short term period. Only a limited number of species, in fact, results stable and able to maintain appropriate demographic levels; instead the most of systematic groups in declining constantly (Zerunian, 2002; Zerunian, 2003).

A big hope for the future lays in European directives addressed to the protection of both water resources and quality of freshwater habitats, *in primis* the Water Framework Directive (Directive 2000/60/EC) and the Habitats Directive (more formally known as Council Directive 92/43/EEC) whose transposition could lead to a significant change in the national culture. Actually, freshwater fauna is consider “minor fauna” by several institutional level but is the basic indicator of the quality of the principal and essential resource to the human survival: the water.

For these reason we started a cooperation with a foreign company, Cryogenetics AS based in Hamar (Norway), specialized in developing tools and products for enhancing the fertilization rate in aquaculture implants and Hedmark University College (Hamar, Norway) expert in salmonids genetic studies.

These collaborations led to the work behind this dissertation, a study aiming to the conservation of one endemic Italian freshwater species: the marble trout (*Salmo marmoratus,* Cuvier 1817).

***1.1 Salmo marmoratus*, Cuvier (1817)**

1.1.1 Classification and description

According to the IUCN Red List there are 2271 species of fishes extinct in the wild or threatened to different extent (IUCN 2015). In Italy, a high number of freshwater fishes are considered autochthonous taxa (Gandolfi *et al.*, 1991; Zerunian, 2002; Kottelat and Freyhof, 2007) with a high number of endemic and sub-endemic species. In Italy are recorded, for the Salmoniformes order, 3 endemic species including the marble trout (*Salmo marmoratus*).

Marble trout (*S. marmoratus*) is a fish belonging to the order Salmoniformes and the Salmonidae family (Fig. 1).

Figure 1: *S. marmoratus* (marble trout)

Usually this fish can reach 50 to 70 cm in length and 5 kg in weight although have been found individuals 140 cm long and 20 kg heavy (Gridelli, 1936). This trout, unlike other species, has a thinner and spindle-shaped body, a less curved profile and a more grown head. The mouth is big and terminal with strong and well developed teeth. The scales that cover the body are small and the lateral line is straight and clear. The first dorsal fin has its insertion frontally comparing to the ventral fins (Specchi *et al.*, 2004). In 1936 Gridelli conducted meristic analyses (Fig. 2 a, b) and measurements on Italian trouts giving the first indication to identify marble trouts. At present days the reference meristic values are those set by Gandolfi *et al.* (1991).

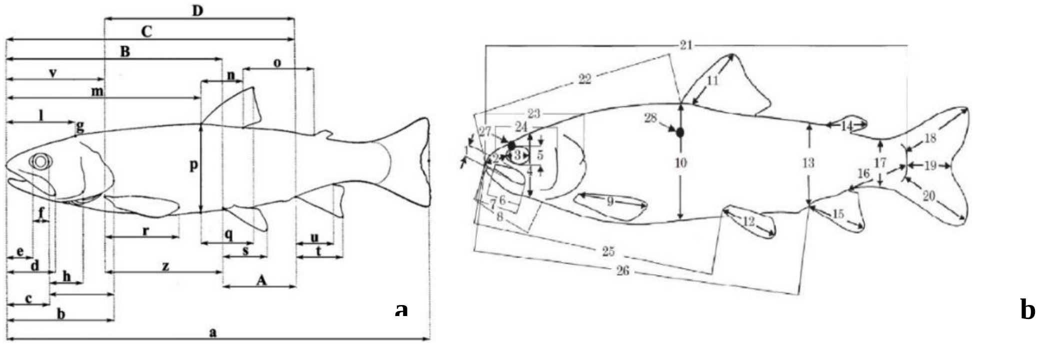

Figure 2 a, b: two schemes illustrating the meristic characters and how to measure them. **a)** Original figure from Gridelli (1936) depicting measurements taken. **b)** Measurements taken on *Salmo* specimens by Delling et al. (2000).

The name of this fish is due to the marbled pattern of his colouration that cover all the body and the head of the trout. In fact, although the colour can show differences *intra-* and *inter-*basins (Fig. 3), this pattern is distinctive and unique of the species.

Figure 3: pics showing differences in the marbled pattern among different river basins. **a)** Adige river basin; **b)** Brenta river basin; **c)** Piave river basin.

Fins are grey with a yellowish shade on the ventral ones; the stomach is white and yellowish too. Some animal populations exhibit small red spot on the body making difficult the species determination (Specchi *et al.*, 2004).

Reproductive season starts approximately in late November and can last, in hatcheries, since early February. Marble trouts reach the sexual maturity around the third/fourth year for females and third year for males. Females can produce up to 1800 eggs per kg of the breeder (Specchi *et al.*, 2004).

1.1.2 Distribution and habitat

This species is an italian sub-endemism and its range of distribution is in the northeast Po river system (Fig. 3) (Meraner *et al.*, 2007).

Figure 3: distribution area of marble trout. In purple the rivers where *S. marmoratus* is present actually.

In Italy marble trout can be found in the left tributaries of the Po river in particular in water basins belonging to Veneto’s estuary like Adige, Brenta, Piave, Tagliamento, Isonzo and other minor rivers. Marble trout presence is recorded also in the Adriatic river systems of western Balkans, in particular in Dalmatia, Montenegro and Albania (Povz *et al.*, 1995).

S. marmoratus can be found in the upper-medium course of a river but the more advantageous habitat is the valley floor course. This species prefers clear and fresh waters, with temperature lower than 16°C, rich in oxygen. Different life stages of marble trout need different habitats: juveniles are more abundant in riffle and run zones with high flowing where they can shelter from predators; adults are more frequent in pools with moderate flowing (Gentili *et al.*, 2001). This species, although it does not migrate so far, can live also in lake basins from where it goes back upstream to reach the reproduction site (Specchi *et al.*, 2004).

1.2 Conservation status and hybridization issue

The 2001 IUCN Red List included the marble trout, already listed in Annex II of EU Habitat Directive (92/43/EEC), in the Least Concern (LC) category. Despite this evaluation, in the last decades the management of this species was not effective causing a drastic decline in the number of individuals, especially in the Italian rivers (Meraner *et al.*, 2007; 2008). According to Gridelli (1936), since the beginning of the 20th century, the brown trout (*Salmo trutta*) has been introduced in the habitat of marble trout. The result has been an overlapping of distribution range resulted into a hybridization between this two Salmonid species. Gridelli conducted in 1936 the first meristic analyses and measurements on Italian trouts and identified *S. dentex*, a species previously described by Heckel (1852), as a distinct species. Two years later Karaman (1938), thanks to Gridelli's work, claimed that *S. dentex* was a hybrid *S. marmoratus* X *S. trutta*. This hybridization ended up at present days with a high level of genetic introgression (Giuffra *et al.*, 1994; 1996; Meraner *et al.*, 2007; 2010) and a loss of genetic variability (Berrebi *et al.*, 2000; Fumagalli *et al.*, 2002; Jug *et al.*, 2005; Meldgaard *et al.*, 2007). New evaluations performed in 2015 by the Italian IUCN Red List predict a future decline of the 80% for the marble trout because of both the habitat alteration and the introduction of brown trout, changing the LC assignment in Critically Endangered (CR) (Rondinini *et al.*, 2013). The native marble trouts are already rare in most rivers and this determines a low number of breeders in the wild and a low production of pure marble trout offspring every year. The combined approach involving morphologic examinations and genetic investigation ensure a high level of selection of the pure marble trout that has to be bred in the fish farm and directed to the reproductive career. The trouts were analyzed combining the *D-Loop* variation in mitochondrial DNA haplotypes and RFLP (Restriction Fragment Length Polymorphism) in nuclear DNA (*LDH-C1**) (Bernatchez *et al.*, 1992; Patarnello *et al.*, 1994; McMeel *et al.*,2001; Nonnis Marzano *et al.*, 2003; Apostolidis *et al.*, 2007). These two basic approaches were combined by Chiesa *et al.* (2016) with genotyping highly polymorphic AFLP (Amplified Fragment Length Polymorphism) loci (Papa *et al.*, 2005; Maldini *et al.*, 2006; Chiesa *et al.*, 2011) to increase the resolution power of the analyses in detecting hybrids. Now in this work we developed, in partnership with Hedmark

University College (Hamar, Norway), a panel of 15 microsatellite loci to investigate further the population structure of 86 putative pure marble trouts, the allele richness and the genetic diversity. Using these additional investigations allow us to choose the breeders with the higher genetic variability for the subsequent reproductive season.

1.3 New technologies for the species conservation

For the reason cited above it is required to change the management and restocking strategies, to focus on the selection of the animals already present in hatcheries in order to leave the wild breeders in rivers and to increase the offspring production in captivity.

Selection of the best breeders, both for the genetic variability and the semen quality, is important to the fish farms that supports conservation programs because it is of interest to increase the efficiency of artificial fertilizations and the number of the fish born in every reproductive season (Kjørsvik *et al.*, 1990; Bromage and Roberts, 1995). Since the 1960's several authors have studied characteristics of salmonids sperm like morphology, motility, seminal plasma parameters, sperm concentration and metabolism (Hwang and Idler, 1969; Christen *et al.*, 1987; Aas *et al.*, 1991; Ciereszko and Dabrowski, 1993; Lahnsteiner *et al.*, 1998; Dietrich *et al.*, 2005). All these individual factors added together with anthropogenic interferences like rearing condition, different methods to collect and store milt, temperatures and condition for sperm activation and frequent fish handling, can induce variation in sperm quality. Since some parameters are hard to measure on field because of the lack of certain equipment and the time needed to do all the measuring in the time between milt collection and artificial fertilizations, in this study we'll assess just the motility and sperm concentration.

Another useful tool in conservation programs is the cryopreservation of gametes (Elder & Brian, 2000; Suquet *et al.*, 2000; Cabrita *et al.*, 2010) both for store milt, facilitate the reproduction in fish farms and for conserve the gametes of the best breeders selected. Blaxter attempted this technique for the first time in 1953 and, in the following decades, has been improved (Mazur, 1964; Ashwood *et al.*, 1980; Felix, 1985; Kumai *et al.*, 1998) becoming a secure and a well investigated procedure in many countries. In cryopreservation, samples are freezed in liquid nitrogen following specific protocols that bring the temperature to -191°C without damaging the cells (Leung, 1991; Dobrinsky 1996; Martino *et al.*, 1996a; Martino *et al.*, 1996b; Isachenko *et al.*, 1998; Zeron *et al.*, 1999). In Italian aquaculture, however, this procedure is not common because of the supposed high cost and because in the country there are not companies that provide this service for fishes. Actually, the budget required in order to send and store milt in a multinational company is not expensive and could be very helpful in freshwater conservation biology.

For this reason we started a cooperation with the foreign company, Cryogenetics AS based in Hamar (Norway), specialized in developing tools and products for enhancing the fertilization rate in aquaculture implants.

2. Aims of the thesis

The aim of this work is to select genetic strains of marble trout in order to breed them in a selected hatchery whose work is focused mainly in fish species conservation. Integrating genetic analyses and innovative approaches in artificial insemination can lead to the accomplishment of a live gene-bank within a hatchery program whose aim is to breed selected fish for restocking purposes (Bjoru & Garseth, 2009). In Norway these hatcheries are a common practice in order to preserve several strains of *Salmo salar*, one of the principal economic resource of the country, selected at geographic level and free from *Gyrodactylus salaris* infections a protozoan threatening Atlantic salmon populations (Hytterød *et al.*, 2015). This model has proved to be successful: animals are selected genetically, stocked separately according to different strains/basins, artificial fertilizations are never carried out mixing animals from different river basins and the offspring are released in the wild.

Our aim is trying to reproduce this successful system in Italy adding the milt cryopreservation process to genetic characterization of the broodstock. The combined approach involving morphologic examinations of milt to assess sperm quantity and quality, its cryopreserved storing coupled to genetic investigations ensure a high level of selection of the pure marble trout that has to be bred in the fish farm and directed to the reproductive career. Being able to cryopreserve milt can be useful in order to limit males presence in hatcheries. Less males is equal to more space in tanks for females fish and less territoriality fighting consequently leading to lower cost for their maintenance, less antibiotics and medicine for animals. The milt cryopreservation implication has both an impact on wild stocks, because of the release of males in rivers after the stripping, and on animal welfare in hatcheries.

In my dissertation I present a track from the selection of the fish in the wild to the cryopreservation of gametes.

3. Materials & Methods

3.1 Collecting samples

In total, 229 breeders of *S. marmoratus* were collected from the hatchery Centro Ittico Valdastico in the Veneto region, North Italy (Appendix A; Fig 4a). Sixty trouts were born in this ichthyogenic center while 84 breeders were donated to this hatchery by Associazione Bacino Acque Fiume Brenta (Bassano del Grappa, VI) and 85 animals previously born from wild captured breeders of Piave river were a gift by hatchery Bolzano Bellunese (Belluno, BL). The 60 putative marble trout of Valdastico hatchery came from three different river basins: Adige, Piave and Brenta (Table I; Fig. 4b) and were captured by electrofishing using a backpack model electrofisher (EnginePowered Electrofisher ELT6011, 300/500V Max, 1300W, Honda engine, Han-grass,Germany) applying pulsed direct current (Fig.5 a, b).

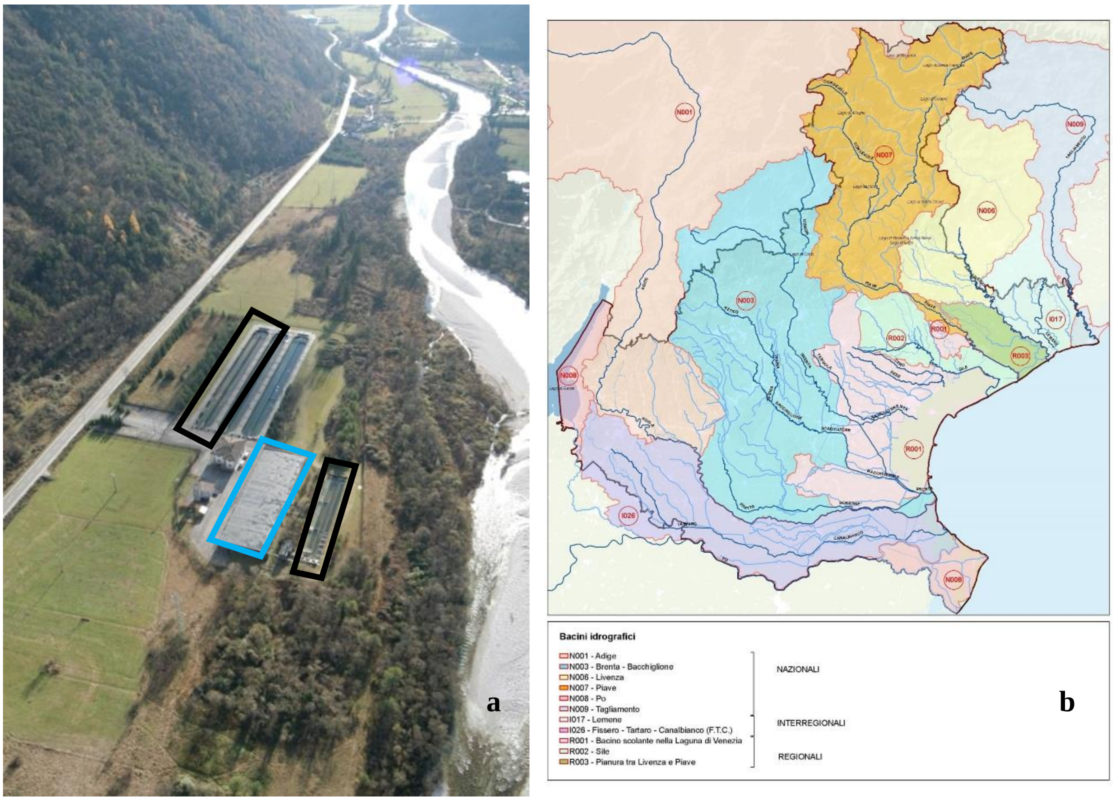

Figure 4: **a)** aerial view of the Centro Ittico Valdastico managed by Veneto Agricoltura, (VI). In the black rectangles are highlighted the outdoor tanks where marble trouts breeders are kept; in the light blue rectangle is in evidence the shed where are locate indoor tanks and the hatchery for eggs and fry. **b)** map showing the main river basins in Veneto region. Marble trout breeders came from basins number: in light pink the Adige river N001, in light blue Brenta-Bacchiglione river N003 and in orange Piave river N007 (Image from ARPA Veneto website).

Table I Collection sites of *Salmo marmoratus* breeders. Fish farm, river basins and number of collected samples (N) are provided.

Fish farm	*River basin*	*N*	*Abbreviation*
Valdastico	Adige	20	AdV
Valdastico	Piave	20	PiV
Valdastico	Brenta	20	BrV
Bassano del Grappa	Brenta/Cismon	84	BrBG
Belluno	Piave	85	PiB

Figure 5: **a)** ichthyologists sampling by electrofishing with backpack model electrofisher **b)** EnginePowered Electrofisher ELT6011, 300/500V Max, 1300W, Honda engine, Han-grass,Germany

Fishes, before the transfer in the hatchery, were submitted to a strict phenotypic selection based on some peculiar morphological traits (Gandolfi et al., 1991) mainly regarding external pigmentation, presence/absence of red spot. Those selected, were tagged with an intramuscular passive integrated transponder (Biomark FDX-B PIT tags) to identify them in the hatcheries. Each PIT tag has a different barcode number that can be read by a proper reader (Fig.6).

For every breeder transferred in the hatchery are recorded some data like sex, weight, length and personal barcode (data in Appendix A; Fig.7).

Figure 6: Biomark HPR Plus™ reader used in Centro Ittico Valdastico (VI) to read PIT-tag barcodes of the marble trout breeders.

In the hatchery the breeders are kept in different tanks depending on their river basin of origin.

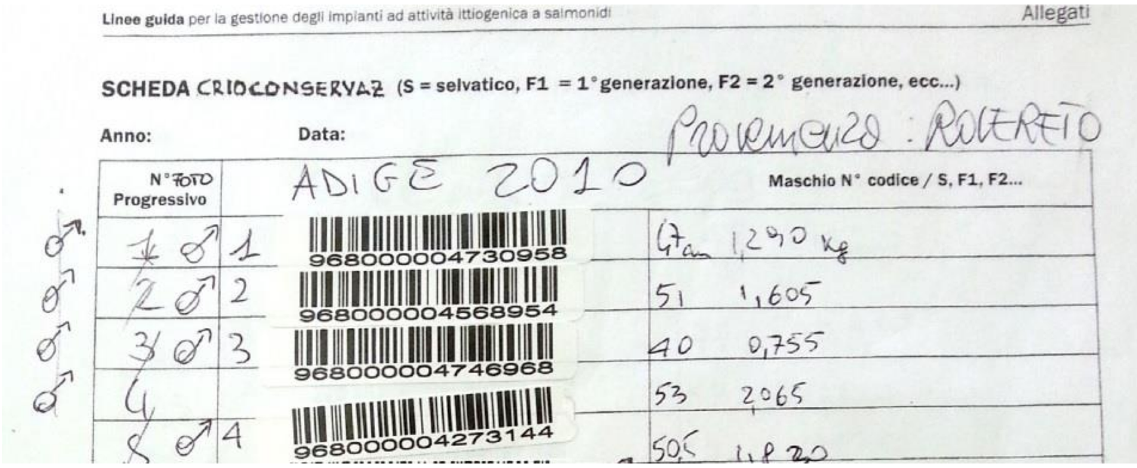

Figure 7: example of a datasheet from Centro Ittico Valdastico (VI). In the first column the progressive number of the picture and the fin sample taken from the individual and the sex; in the second column the sticker of the PIT tag barcode with the identification number; in the third column the length expressed in cm and the weight express in kilograms. In the table also the sampling site (Rovereto), the river basin (Adige) and the year of collection (2010).

As outgroup were collected 24 *Salmo cettii* (Mediterranean trout) from a hatchery in Santa Fiora (GR) near mount Amiata in Tuscany (Fig. 8).

Figure 8: operators and volounteers gathering Mediterranean trout breeders by seine net in the *S. cettii* tank in Santa Fiora (GR).

The sampling collection for genetic analyses consisted in cutting a small portion of the adipose fin in order to avoid the breeder sacrifice. The adipose fin is a soft, fleshy fin found on the back behind the dorsal fin and just forward of the caudal fin. The cut does not produce bleeding so it is not harmful for the animal. Scissors and tweezers were sterilized with ethanol between samplings to avoid contamination. Every fish, after sampling, was released in the tank corresponding to the basin of origin. The fin samples are stored at -20°C in absolute ethanol in the Laboratory of Molecular Zoology, Department of Chemistry, Life Sciences and Environmental Sustainability, University of Parma (Italy).

3.2 DNA isolation

High molecular weight genomic DNA was isolated and purified from ethanol-fixed fin tissue samples stored at -20°C. DNA was isolated and purified using Wizard® Genomic DNA Purification Kit (Promega) following the manufacturer instructions. DNA quality was inspected by visualization on 1% agarose gel electrophoresis in TAE buffer. All DNA samples are stored at -20°C, at the Laboratory of Molecular Zoology, Department of Department of Chemistry, Life Sciences and Environmental Sustainability, University of Parma (Italy).

3.3 Mitochondrial analyses of the D-loop

The D-loop region (mitochondrial control region) was amplified following the method of Apostolidis *et al.* (2007) by using a single common reverse primer (CMOD-REV, Eurofins

Genomics) and four forward lineage-specific primers (278C, 41T, 212C and 128A, Eurofins Genomics). A reaction volume of 30 µl containing 1 U of GoTaq (Promega), 1.5 mM Mg2+, 0.2 mM dNTPs and 10 pmol of each primer was used. Multiplexes were performed using the following conditions: an initial 3 min denaturation step at 94°C, 35 three-step cycles of 10 s at 94°C, 10 s at 47°C and 20 s at 72°C, followed by a final extension at 72°C for 10 min. PCR products were visually analyzed on 2.5% agarose gel electrophoresis in TAE buffer. In every PCR were run four positive controls (Fig.9) and a negative control in order to check both the success of the amplification reaction and the absence of contaminations. This marker is among the few ones able to discriminate marble trout haplotype from other three haplotypes: Adriatic, Atlantic and Mediterranean (Bernatchez *et al.*, 1992; Dovc *et al.*, 2004). Individuals that present marble haplotypes were submitted to nuclear DNA analyses.

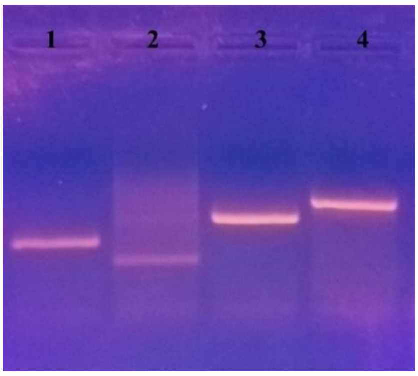

Figure 9: agarose gel displaying the four positive control used in the D-loop gene amplification. In the line 1 (approximately 200bp) the Mediterranean haplotype, in the line 2 (approximately 150bp) the Adriatic haplotype, in the line 3 (approximately 300bp) the marble haplotype and in the line 4 (approximately 400bp) the Atlantic haplotype.

3.4 Nuclear LDH-C1 analyses*

The nuclear gene LDH- C1* was amplified using RFLP (Restriction Fragment Length Polymorphism) method and primers Ldhxon3F/Ldhxon4R (Eurofins Genomics) described in McMeel *et al.* (2001). A reaction volume of 16 µl containing 1 U di GoTaq (Promega), 1.5 mM Mg2+,0.2 mM dNTPs and 10 pmol of each primer was used. PCR product were obtained with the following conditions: an initial 5 min denaturation step at 95°C, 30 three-step cycles of 1 min at 95°C, 1 min at 65°C, and 1 min at 72°C, followed by a final extension at 72°C for 10 min. Amplicons were incubated with 1.5 U of BslI for 2 h at 55°C and analyzed by 2.5% agarose gel electrophoresis. The restriction patterns resulting from these analyses has the power to distinguish the heterozygote hybrids from homozygote Atlantic or Mediterranean samples. A *90/90 allele represents the Atlantic taxa, the heterozygote

*90/100 allele identifies a hybrid individual while a *100/100 allele identifies the Mediterranean taxa (Fig. 10). In every PCR were run three positive controls and a negative control in order to check both the success of the amplification reaction and the absence of contaminations.

Figure 10: agarose gel displaying the three positive control used in the LDH-C1* gene amplification. In the line 1 the Mediterranean *90/90 allele, in the line 2 the heterozygote *90/100 allele that identify a hybrid individual, in the line 3 the Atlantic *100/100 allele.

3.5 Microsatellite analyses

3.5.1 Microsatellite panel on Centro Ittico di Valdastico (VI) marble trouts

A panel of 12 microsatellite loci (Angers *et al.*, 1995; O'Reilly *et al.*, 1996; Presa & Guyomard, 1996; Grimholt, 1997; King *et al.*, 2005; Thorsen *et al.*, 2005; Lerceteau- Kohler & Weiss, 2006; Moen *et al.*, 2009; Pujolar *et al.*, 2011; Appendix B, Panel I and Panel II) was tested on the 56 marble trouts from Centro Ittico di Valdastico (VI) in order to investigate the genetic variability of the individuals before the reproductive season. They were amplified in two different multiplexes, considering the size range of the loci, using HOT FIREPol® DNA Polymerase by Solis Biodyne. A reaction volume of 10 µl containing 10X B1 buffer, 25 mM of $MgCl2$, 10 mM of dNTPs, 10mg/mL of BSA, 5X Primer mix and 1U of HOT FIREPol® DNA Polymerase. PCR product were obtained with the following conditions: an initial 10 min denaturation step at 95°C, 35 three-step cycles of 30 sec at 95°C, 1 min at 60°C, and 1 min at 72°C, followed by a final extension at 72°C for 45 min. Amplicons were analysed with Applied Biosystems 3130xl Genetic Analyzer and the output data were examined with GeneMapper® Software v.4.0 (Applied Biosystems, UK; Fig. 11).

3.5.2 *Microsatellite panel on total dataset of trouts*

Considering the results obtained with the 12 microsatellite panel it was decided to expand the analyses to a subset of the marble trouts from the Bassano del Grappa and Belluno hatcheries and the *Salmo cettii* specimens. Nine microsatellite loci (Angers *et al.*, 1995; O'Reilly *et al.*, 1996; Presa & Guyomard, 1996; Grimholt, 1997; King *et al.*, 2005; Thorsen *et al.*, 2005; Lerceteau- Kohler & Weiss, 2006; Moen *et al.*, 2009; Pujolar *et al.*, 2011; Appendix B: Panel IIb and III) were added to the previous panels, for a total of 21 loci, and were tested on a subset of 86 marble trouts and 24 Mediterranean trout. 56 marble trouts came from Centro Ittico di Valdastico hatchery, 15 from Belluno hatchery and 15 from Bassano del Grappa ichthyogenic center. They were amplified in four different multiplexes (Panel I, Panel II, Panel IIb and Panel III; Appendix B) considering the size range of the loci, using HOT FIREPol® DNA Polymerase by Solis Biodyne. A reaction volume of 10 µl containing 10X B1 buffer, 25 mM of $MgCl_2$, 10 mM of dNTPs, 10mg/mL of BSA, 5X Primer mix and 1U of HOT FIREPol® DNA Polymerase. PCR product were obtained with the following conditions: an initial 10 min denaturation step at 95°C, 35 three-step cycles of 30 sec at 95°C, 1 min at 60°C, and 1 min at 72°C, followed by a final extension at 72°C for 45 min. Amplicons were analysed with Applied Biosystems 3130xl Genetic Analyzer and the output data were examined with GeneMapper® Software v.4.0 (Applied Biosystems, UK; Fig. 11).

Figure 11: example of microsatellites results for one sample of the dataset. In the screenshot are displayed results for the Panel I. In every line the microsatellites peak divided according to the fluorophore used for the marker in the multiplex (Appendix B).

3.6 Statistical analyses for genetic data

Mitochondrial and LDH-C1* data, well-standardized markers for Mediterranean salmonids, allow to discriminate putative pure marble trouts from hybrids and non-native trouts. Data were analysed as frequencies of haplotypes and genotypes (Table II).

The 12 microsatellite panel analyses were used to evaluate the allelic diversity of the breeders in order to identify possible mating matches for the reproductive season in Centro Ittico di Valdastico. The 21 microsatellite panel test was performed both in order to verify if the hatcheries made a good work in maintaining the trouts divided during the reproductive season, according to their river basins of origin, and the potential presence of hybrids in the dataset.

Microsatellites data were analyzed by Genetix software (Belkhir *et al.*, 1996-2002), which provided a Factorial Correspondence Analysis (FCA), allelic richness tables and Nei's genetic distance.

Using STRUCTURE version 2.3.4 (Pritchard *et al.*, 2000) was conducted, on the two different microsatellite panel dataset, a Bayesian clustering analysis. The parameters used were: 100,000 burn-in and 100,000 Markov chain steps and admixture model with independent allele frequencies; each simulation was performed for K values ranging from 1 to 10. STRUCTURE HARVESTER Web v0.6.94 (Earl and vonHoldt, 2012) was used in order to evaluate the highest level of population structure; both the mean posterior probability of the data [Ln(K)] and the ΔK (Evanno *et al.*, 2005) were calculated to estimate the number of the population (K) based on microsatellite dataset. Structure assignment tests were then performed according to the most probable K values.

3.7 Motility and milt concentration analyses

Sperm motility and milt concentration were measured in the hatchery during the reproductive season from the 23rd of November 2015 until the 3rd of February 2016. PIT tags of male breeders were checked before the stripping operations; according to the genetic analyses only the pure marble trouts and, where possible, the individuals presenting highest degree of genetic variability were chosen. Animals were sedated in a 30% phenoxyethanol solution and stripped manually by operators (Fig. 12 a, b). Milt was collected in syringes without needle to avoid contaminations, or in tissue culture flasks, preserved in ice and analyzed in the laboratory.

Figure 12: **a)** marble trout male breeder sedated in a 30% phenoxyethanol solution; **b)** operators in Centro Ittico di Valdastico hatchery stripping manually a marble trout male breeder and collecting milt in a tissue culture flask for the subsequent laboratory analyses.

3.7.1 Motility assessment

Typically, the motility evaluation is assessed *via* computer-assisted sperm analysis (CASA) systems or cell motility analysis (CMA). These techniques, well known since the 1980 are developed to evaluate several characteristics of the spermatozoa motility such as speed, direction etcetera (Cosson *et al.*, 1997; Kime *et al.*, 2001; Rurangwa *et al.*, 2004; Dietrich *et al.*, 2005). In my dissertation work, I performed visual analyses with a phase-contrast or dark-field microscope (Billard *et al.*, 1977, 1995; Cosson *et al.*, 1999; Ingermann *et al.*, 2002; Christen *et al.*, 1987). 10 µl of AquaBoost® Activator (Cryogenetics® and Minitüb GmbH) were placed on a slide and a small drop of milt was mixed to it (Fig.13). Values assessed for motility ranged from 0 to 3: 0 for no motility at all or only few spermatozoa moving, 1 for the 20-40% of spermatozoa moving, 2 for 50-70% of spermatozoa moving and 3 for 80-100% of spermatozoa moving.

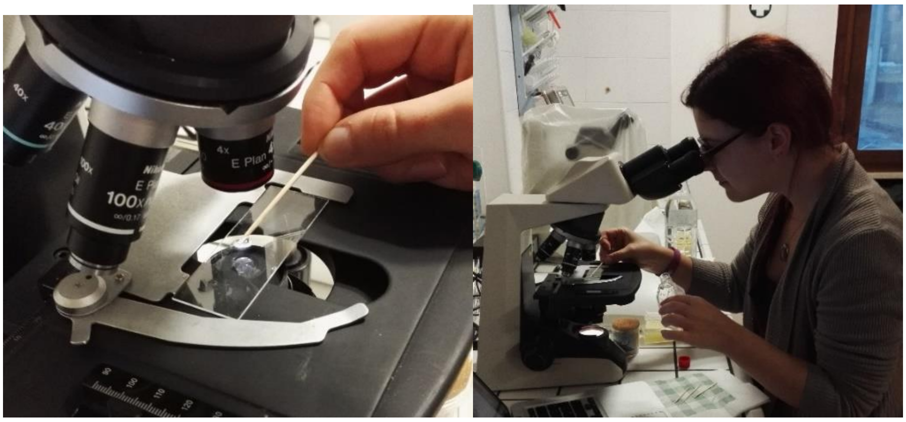

Figure 13: visual analyses with a phase-contrast or dark-field microscope. A small drop of milt from the culture flask was mixed, using a toothpick, with 10 µl of AquaBoost® Activator (Cryogenetics® and Minitüb GmbH) and placed on a slide for the motility assessment.

3.7.2 Concentration assay

The sperm concentration in each sample of milt was measured with photometer SDM6 (Minitüb GmbH and Cryogenetics®, Fig.14). Tipically sperm concentration is measured by spectrophotometric method of Ciereszko and Dabrowsky (1993) standardized by counting the sperm density in a cell counting chamber (Neubauer, Makler, Burker or Thoma chambers) and with spermatocrit determination (Foote, 1964). The photometer used in this work was developed for measure the dimensions of spermatozoa of different animals, including salmonids, for a more reliable evaluation of the sperm concentration in each sample. For the measuring were used 10 µl of milt diluted in 4 ml of NaCl 0.9% (Sodio Cloruro EUROSPITAL) in polystyrene disposable cuvettes (Sarstedt). A solution of 0.9% NaCl served as a blank.

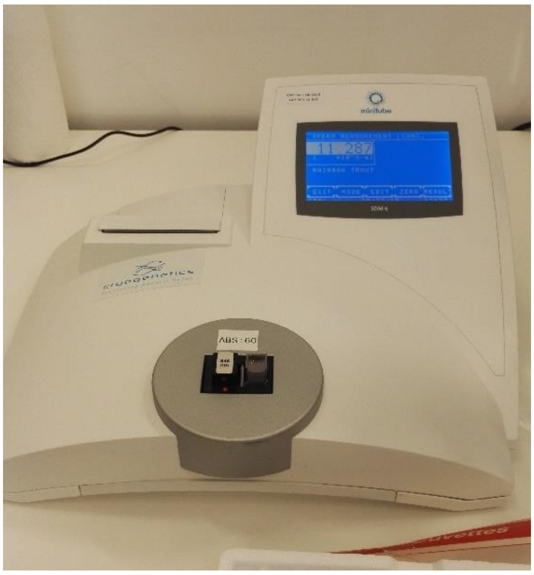

Figure 14: photometer SDM6 (by Minitüb GmbH and Cryogenetics®) used for the sperm concentration assay.

3.8 Concentration during reproductive season

During all the reproductive season, from the 23rd of November 2015 until the 3rd of February 2016, nine males were kept separated in the same outdoor tank in order to measure every week the variations in milt concentration. This analysis was conducted in order to identify the time span when the milt concentration was maximum. Since the frequent manipulation and sedation can be harmful for fishes, operators decided to use also hybrid individuals for this experiment (Table III). Three individuals for each river basin were chosen randomly and exposed to the same treatment for the stripping described in paragraph *2.7*. Both motility assessment analyses and concentration measurement were performed as described in paragraph *2.7.1* and *2.7.2*.

Table III: table displaying the individuals randomly chosen for the measure of the milt concentration through reproductive season. In the first column the fish-ID composed by the last four number of the barcode; in the second column the river basin of origin; in the third column the genotype (H=hybrid, P=pure marble trout).

Fish-ID	Basin	Genotype
4735	Piave	H
8099	Piave	H
8431	Piave	P
7234	Brenta-Valsugana	H
7202	Brenta-Valsugana	P
9111	Brenta-Valsugana	H
5900	Adige	P
5379	Adige	P
6504	Adige	P

One-way ANOVA two-tailed with Tukey's post test was performed using GraphPad Prism version 7 (GraphPad Software) in order to test if there was a statistically significant difference between animals belonging to different river basins and an unpaired t test two-tailed with Welch's correction to test differences between pure marble trout and hybrids.

3.9 Artificial fertilization

3.9.1 Egg fertilization experiment

During the reproductive season (November 2015/February 2016), was conducted an experiment to test if there was a difference between the use of non-diluted milt vs the use of diluted milt in terms of fry production. Data of motility and milt concentration were collected in an Excel data sheet created for the purpose by the company Cryogenetics® (Fig. 15). The algorithm calculated the right amount of AquaBoost® Dilutor that must be added to the fresh milt sample. Once the dilutor was added to the sample in the tissue culture flask with a graduated cylinder the milt was stored at +4°C on a rocker shaker (BioSan Mini Rocker-Shaker MR-1) until the fertilization.

Dilution of milt - AquaBoost® Dilutor

cryogenetics

AquaBoost Milt Dilutor

Sperm concentration after dilution	2	*10^9/ml
Total # eggs to fertilize	300	
Sperm-to-egg ratio	3,5	million(s)/egg
Total volume diluted milt required	0,5	ml
=	0,000525	litres

Accumulated: 556 ml

Date	Fish ID	Motility	Contamination	Concentration (10^9/ml)	Milt volume (g)	Volume AquaBoost Dilutor (g)	Total volume (g)	Comments
08-12-15	801	3	nessuna	15,01	2	**13**	**15**	Piave
08-12-15	725	3	nessuna	20,95	4	**38**	**42**	Piave
08-12-15	868	2	nessuna	12,59	2	**11**	**13**	Piave
08-12-15	356	3	nessuna	16,53	4	**29**	**33**	Piave
08-12-15	431	2	nessuna	14,66	1,5	**9**	**11**	Piave
08-12-15	785	3	nessuna	15,98	1,5	**10**	**12**	Piave
08-12-15	53	3	nessuna	13,90	2,5	**15**	**17**	Piave
08-12-15	610	2	nessuna	17,21	2	**15**	**17**	Piave
08-12-15	730	3	nessuna	12,69	1,5	**8**	**10**	Piave

Figure 15: example of Excel data sheet used in the dilution of milt. In the higher box the operator must insert the fertilization condition like the sperm concentration that wants to obtain after dilution, the total number of eggs to fertilize and the sperm-to-egg ratio. In the yellow box the algorithm result given in ml of diluted milt that has to be used. In the columns the operator has to write the date of sampling, the fish ID, the motility assessment, the contamination, the concentration measure obtained with SDM6, the milt volume obtained from the stripping. In the two following columns, in bold, the amount of dilutor that the operator must add to the fresh milt.

During the season were performed 7 fertilizations:

1. 23/11/2015 – Piave and Brenta;
2. 09/12/2015 – Piave and Brenta;
3. 15/12/2015 – Adige;
4. 23/12/2015 – Piave and Brenta;
5. 05/01/2016 – Piave, Brenta and Adige;
6. 20/01/2016 – Brenta;
7. 03/02/2016 – Brenta and Adige.

After the laboratory analyses on the milt, the operators proceeded with the stripping of the females marble trout breeders. As the operations made for males, fishes were sedated in a 30% phenoxyethanol solution and stripped manually. Eggs obtained were collected in a bucket, rinsed with fresh water to eliminate the ovarian liquid and divided in plastic plates. For every experiment were used an amount of about 600 eggs, divided in two plates, obtained from a pool of various females (Fig.16): 300 eggs were fertilized with fresh milt, 300 with diluted milt from the same male.

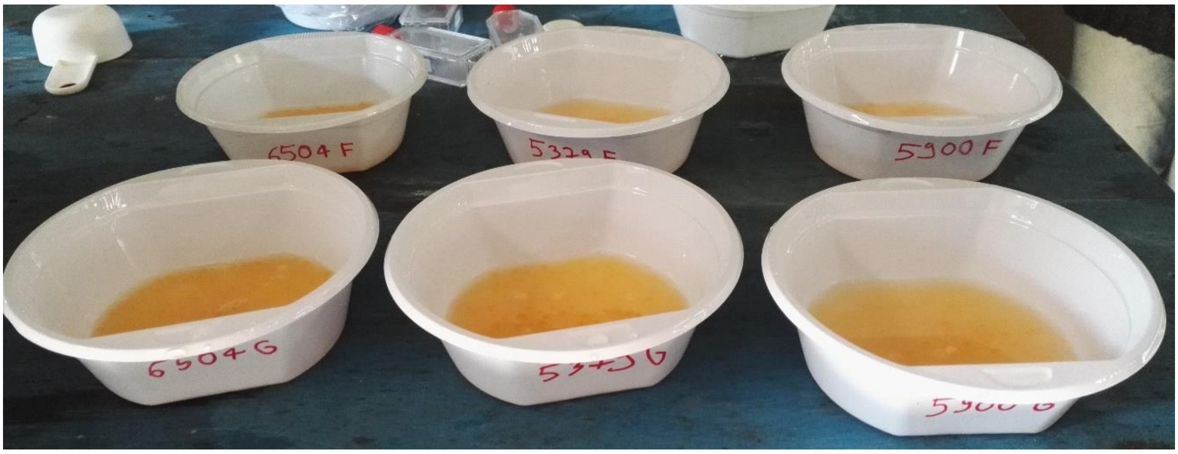

Figure 16: plastic plates used in the egg fertilization experiment. On each plate is written the four last number of the barcode of the sire and the initial of the name of the operator that fertilized the eggs in order to identify the two treatment (diluted and non-diluted milt).

Both the milt samples, for every experiment, were of the same breeder and were analysed before the use. In order to activate sperm were used, respectively, water from the tanks and AquaBoost® Activator. After the fertilization, and the subsequent 5 minute incubation, eggs were rinsed with water in order to eliminate the dead ones and the remaining milt and incubated in a vertical incubator (Fig.17 a,b). Every small box in the incubator had a card reporting the fertilization data, the river basin and the barcode of the male used to fertilize that egg stock.

Figure 17: **a)** eggs are rinsed with water in order to eliminate the dead ones and the remaining milt; **b)** eggs in metal boxes stocked in one drawer of the vertical incubator.

Operators counted the dead eggs during the normal procedure of removal from the incubator, in order to prevent fungal infections. The number of dead fry and eggs was subtracted from the total number of eggs fertilized.

3.9.2 Statistical analyses for the egg fertilization experiment

In order to investigate if the differences between the fertilization with pure and diluted milt were significant was used the Pearson's chi-squared test χ^2 (Pearson, 1900). Software R, version 3.2.1 GUI 1.66 Mavericks build (6956; R Core Team, 2015), was used to run the test: the matrix was 2x2 and included the number of dead eggs and born fishes using both the diluted and non-diluted milt. According to the limited number of samples analysed Yate's correction was also considered. Null hypothesis stated that differences in hatched eggs rate were due to coincidence while the alternative hypothesis claimed that that difference was due to a different yield in the use of non-diluted *versus* diluted milt.

3.10 Cryopreservation

The milt of the breeders with higher genetic variability and the ones that presented higher motility and sperm concentration values were shipped to Cryogenetics AS based in Hamar, Norway. In specially designed laboratories it was analysed again for motility and concentration upon arrival and prepared for the cryopreservation. The milt was diluted according to AquaBoost® Dilutor data sheet results, a cryoprotectant was added and the sample was packed in labeled storage containers. Each container needs to be tailored to its application; an optimal volume, biosecure, non-toxic, practical, space-saving and aid the fertilization process after thawing. There are two types of containers that can be used efficaciously for salmonids: first is the SquarePack® whose volume is 12.5 ml and is designed for freezing milt in large volumes, in fact it can be used for fertilize 3000 eggs; second is the straw whose volume is 0,5 ml by which can be fertilize an amount of 300 eggs. After the packing the storage containers were freezed in liquid nitrogen and stocked in dewars in the storage rooms.

4. Results

4.1. Mitochondrial haplotyping and LDH-C1 genotyping*

The results of the D-Loop control region (mitochondrial analyses) revealed the percentage of different haplotypes within the entire group of 229 marble trout breeders. Detailed data are presented according to the river basin of origin of the trouts (see Table II). 100% of the individuals displayed marble haplotypes. Adriatic, Mediterranean and Atlantic haplotypes were not found in the dataset.

Regarding nuclear gene LDH-C1* data (Table II), were found the presence of all three different genotypes among the breeders. Frequencies of the homozygote genotype *100/100 (Mediterranean) was 82.53% of the whole dataset while 16.59% of the samples were detected as *90/100 heterozygotes (hybrids) and the 0.87% of samples was homozygote genotype *90/90 (Atlantic).

Table II: Results of mitochondrial and nuclear analyses showing the percentage of different haplotypes (D-Loop) and genotypes (LDH-C1*) within the entire group of 229 marble trout breeders. Me: Mediterranean, Ma: Marble, A: Atlantic, Ad: Adriatic, HE: Heterozygote At/Me.

River basin	N	D-Loop				LDH-C1*		
		Me%	Ma%	A%	Ad%	At%	Me%	He%
Adige	20	0	100	0	0	0	85	15
Piave	105	0	100	0	0	2.1	87.4	10.5
Brenta/Cismon	104	0	100	0	0	0	74	26

4.2 Microsatellite analyses

4.2.1 Microsatellite panel on Centro Ittico di Valdastico (VI) marble trouts

A subset of 56 putative marble trout breeders, previously characterized with mitochondrial and nuclear markers and coming from 3 different river basins, was genotyped by a microsatellite panel. 12 microsatellites, previously tested for *Salmo salar* and *Salmo marmoratus* and present in literature (Angers *et al.*, 1995; O'Reilly *et al.*, 1996; Presa & Guyomard, 1996; Grimholt, 1997; King *et al.*, 2005; Thorsen *et al.*, 2005; Lerceteau- Kohler & Weiss, 2006; Moen *et al.*, 2009; Pujolar *et al.*, 2011; Appendix B), were amplified and the results used to calculate the percentage of presence for every allele in every group. Five loci among the 12 of the panel, BHMS349, Ssa197, SSaD157, SsaD58, STR-2, displayed a higher allelic richness so they were more polymorphic (Appendix C), the locus SsaD170 presented a high amount of null allele between the samples of the dataset so was deleted from the subsequent analyses. Allelic richness per locus in Appendix C.

Data from microsatellite panel were analysed with a Bayesian clustering analysis using STRUCTURE 2.3.4 (Pritchard *et al.*, 2000). Results from the program run were then checked with STRUCTURE HARVESTER Web v0.6.94 (Earl and vonHoldt, 2012) in order to evaluate the highest level of population structure. Adopting a hierarchical approach, the first level of Structure analysis suggested that K=3 (Fig. 18 a, b) was the most likely solution to represent population structuring of the dataset, according both to the parametric and non-parametric tests (Evanno *et al.*, 2005).

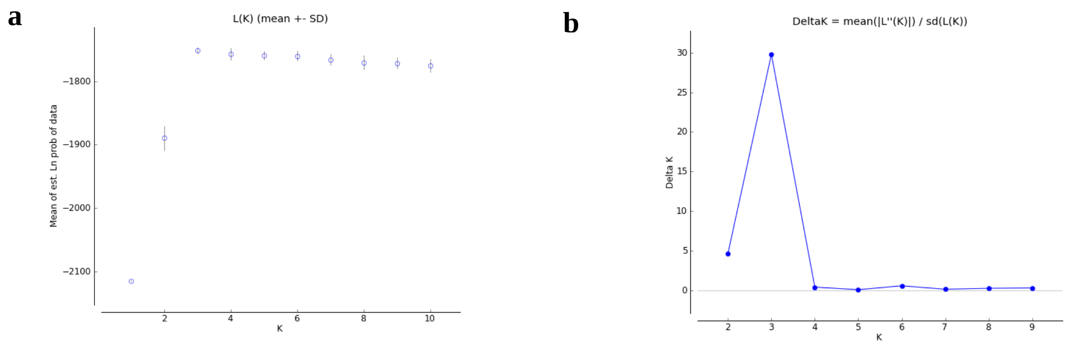

Figure 18: STRUCTURE HARVESTER outputs on the whole microsatellite dataset. (a) Estimated Ln(K). (b) Estimated [ΔK] (Evanno *et al.*, 2005).

Bar plot from STRUCTURE 2.3.4 (Fig. 19) showed a clear division between the tree river basins of origin for the 56 marble trout analyzed. 17 samples belong to q1 cluster corresponding to Adige river; 21 samples belong to the q2 cluster corresponding to Brenta river; 18 samples seems to belong to the q3 cluster corresponding to the Piave river (Zuccon *et al.*, in press).

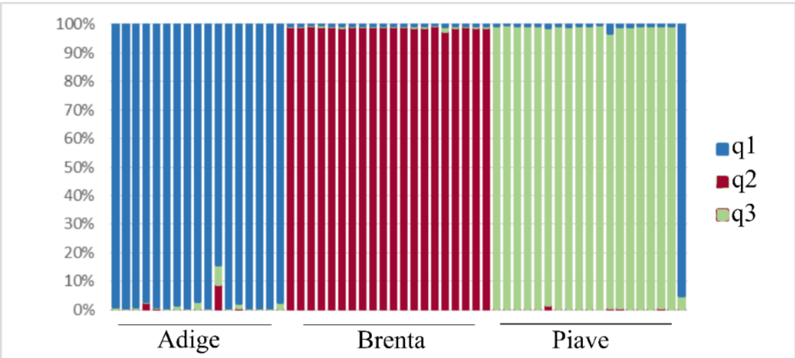

Figure 19: STRUCTURE software analyses on the whole microsatellite dataset. Clustering analysis of the entire dataset for estimated K = 3; q1, q2 and q3 represent the q-values, expressed as probability values of each sample to belong to a specific cluster. On the *x* axe the river basin of origin corresponding to the cluster assignment.

4.2.2 Microsatellite panel on total dataset of trouts

A subset of 86 marble trout breeders, previously characterized with mitochondrial and nuclear markers and coming from 3 different river basins, was genotyped by a microsatellite panel. Thirty marble trouts chosen randomly from the pool of breeders from the hatcheries of Belluno and Bassano del Grappa were added to the 56 pure marble trouts breeders from Centro Ittico di Valdastico. As outgroup were analysed 24 *Salmo cettii* (Mediterranean trout) from a hatchery in Santa Fiora, Tuscany. 21 microsatellites, previously tested for *Salmo salar* and *Salmo marmoratus* and present in literature (Angers *et al.*, 1995; O'Reilly *et al.*, 1996; Presa & Guyomard, 1996; Grimholt, 1997; King *et al.*, 2005; Thorsen *et al.*, 2005; Lerceteau- Kohler & Weiss, 2006; Moen *et al.*, 2009; Pujolar *et al.*, 2011; Appendix B), were amplified and the results used to calculate the percentage of presence for every allele in every group. Allelic richness per locus in Appendix D.

Data from microsatellite panel were analyzed with a Bayesian clustering analysis using STRUCTURE 2.3.4 (Pritchard *et al.*, 2000). Results from the program run were then checked with STRUCTURE HARVESTER Web v0.6.94 (Earl and vonHoldt, 2012) in order to evaluate the highest level of population structure. The web software results suggested that K=4 (Fig. 20 a, b) was the most likely solution to represent population structuring of the dataset, according both to the parametric and non-parametric tests (Evanno *et al.*, 2005).

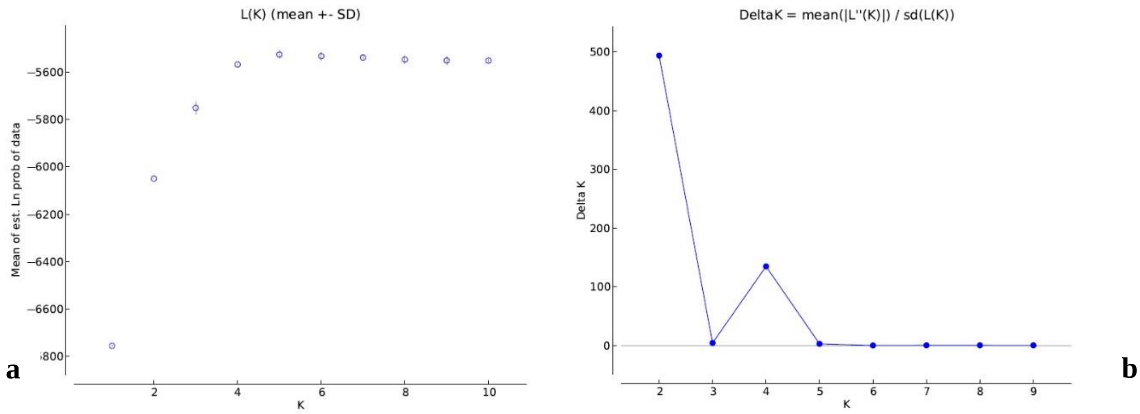

Figure 20: STRUCTURE HARVESTER outputs on the whole microsatellite dataset. **a)** Estimated Ln(K); **b)** Estimated [ΔK] (Evanno *et al.*, 2005).

Bar plot from STRUCTURE 2.3.4 (Fig. 21) showed a clear difference between the samples of the 90 marble trout analyzed and the Mediterranean trout samples.

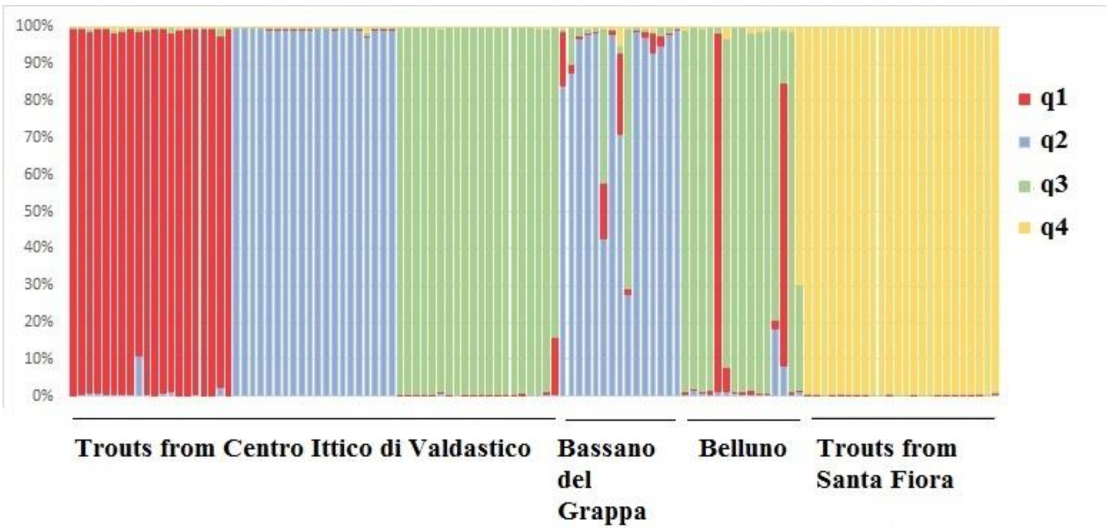

Figure 21: STRUCTURE software analyses on the whole microsatellite dataset. Clustering analysis of the entire dataset for estimated K = 3; q1, q2, q3 and q4 represent the q-values, expressed as probability values of each sample to belong to a specific cluster. On the *x* axe the hatcheries of origin of the trouts: Centro Ittico di Valdastico (VI), Bassano del Grappa (VI), Belluno (BL) and hatchery of Santa Fiora (GR) in Tuscany.

The Factorial Correspondence Analysis (FCA) was conducted with Genetix software (Belkhir *et al.*, 1996-2002) considering every hatchery and every river basin of origin as a single population (Fig. 22). The first axis accounted for 34.20% of total inertia, the second axis for 20.93% and the third axis accounting for 19.21%.

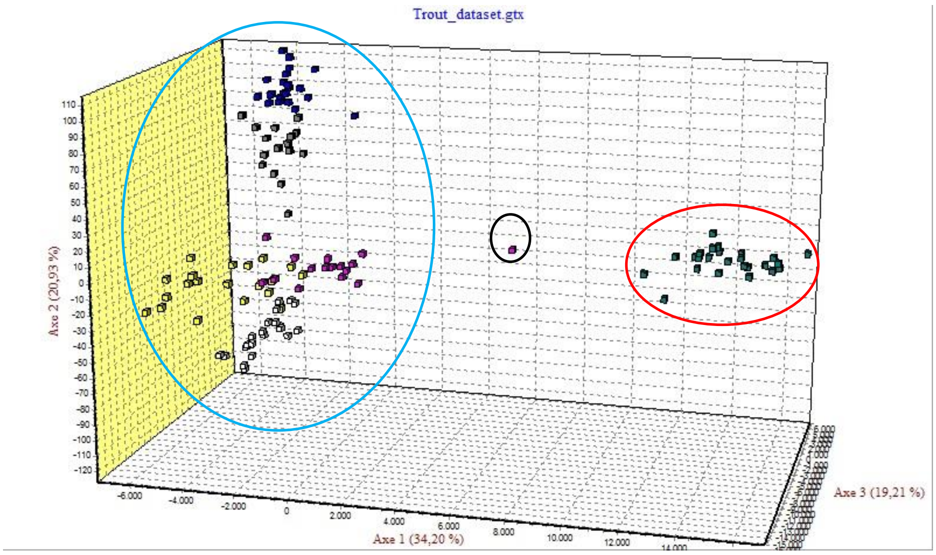

Figure 22: FCA 3D considering every hatchery and every river basin of origin as a single population. In the light blue circle the marble trout breeders; in the red circle the Mediterranean trouts and in the black circle the median individual.

Considering separately the axes were obtained the graphs shown in Fig. 23 a and b. The first axis accounted for 5.80% of total inertia, the second axis for 3.88%. The third axis accounting for 3.63% and the fourth for 3.35% of total inertia didn't show a clear separation between species but rather between the river of origin for the Centro Ittico di Valdastico trouts.

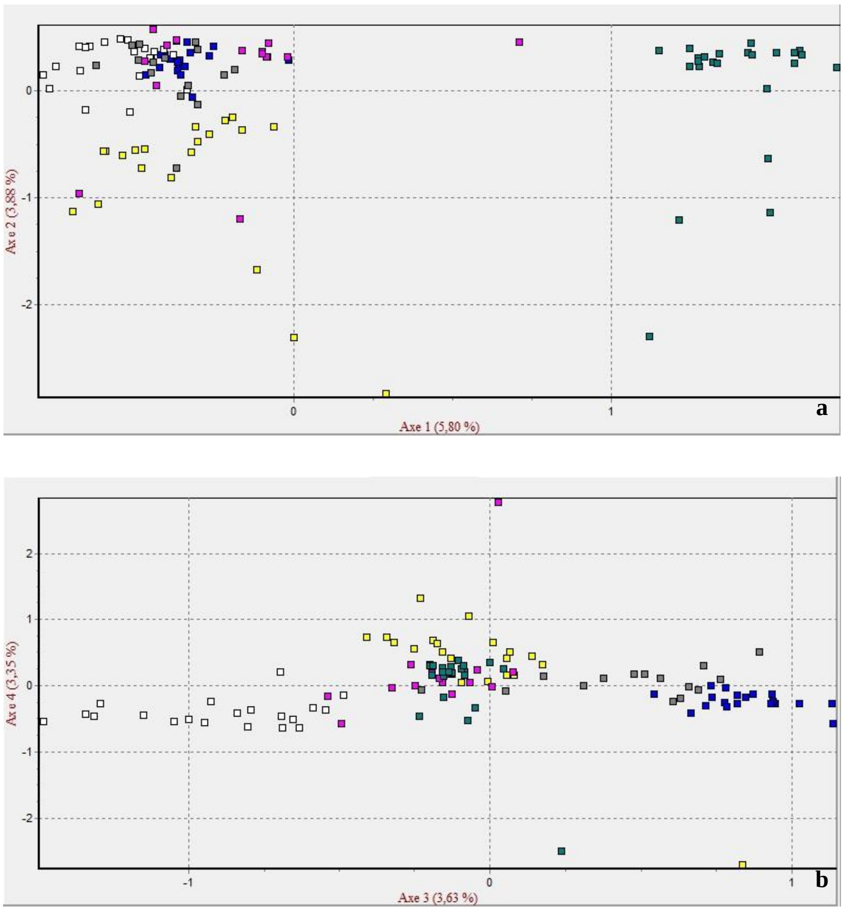

Figure 23: FCA 2D considering every hatchery and every river basin of origin as a single population. **a)** The first axis accounted for 5.80% of total inertia. the second axis for 3.88% **b)** the third axis accounting for 3.63%. and the fourth for 3.35%.of total inertia. In yellow Adige population. in blue Brenta population. in white Piave population from Centro Ittico di Valdastico; in grey Brenta/Cismon population from Bassano del Grappa; in purple Piave population from Belluno; in green Mediterranean trouts from Santa Fiora.

Focusing on Nei's genetic distances based on microsatellite data it is noteworthy that, considering samples belonging to different hatcheries, the lowest values were those obtained by comparison between Bassano del Grappa and Belluno hatcheries (see Table V). The results from the test showed that the higher values of genetic distances were those obtained comparing Mediterranean trouts from hatchery in Santa Fiora to marble trouts coming from the hatcheries in Veneto region.

Table V: Nei's genetic distances based on microsatellite data among analyzed samples. Numbers of analyzed samples is indicated within brackets.

	AdV(20)	BrV(20)	PiV(20)	BrBG(15)	PiB(15)	SF(24)
AdV(20)		0.388	0.376	0.285	0.303	0.855
BrV(20)	0.388		0.496	0.217	0.365	0.848
PiV(20)	0.376	0.496		0.377	0.346	1.154
BrBG(15)	0.285	0.217	0.377		0.240	0.865
PiB(15)	0.303	0.365	0.346	0.240		0.667
SF(24)	0.855	0.848	1.154	0.865	0.667	

4.3 Motility and milt concentration analyses

4.3.1 Motility assessment

The motility assessment was performed *via* visual analyses with a phase-contrast or dark-field microscope. One hundred and thirteen data from various males were collected during the four months of last winter reproductive season. Data of the motility were collected in a barplot (Fig. 24) and were analysed divided per month of sampling. Complete table for motility data can be found in Appendix E.

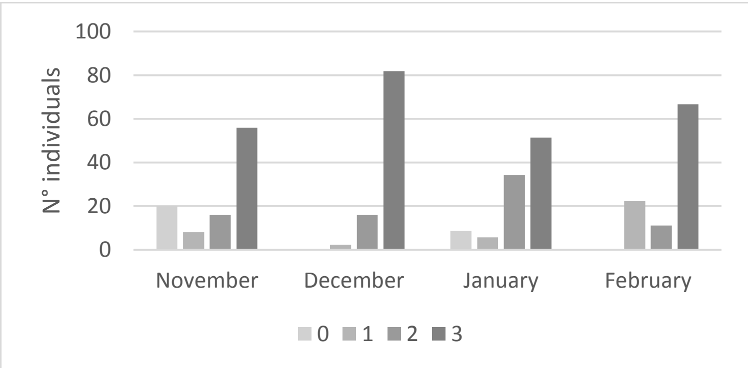

Figure 24: Barplot displaying data of the spermatozoa motility of the 113 males sampled during the reproductive season and divided per month of sampling. 0, 1, 2, 3 indicate the value of motility assessed *via* microscope. 0 for no motility at all or only few spermatozoa moving, 1 for the 20-40% of spermatozoa moving, 2 for 50-70% of spermatozoa moving and 3 for 80-100% of spermatozoa moving.

4.3.2 Concentration assay

The concentration assay was performed *via* photometer SDM6 (by Minitüb GmbH and Cryogenetics®) on the same individuals of the motility assessment the same day of the stripping, output results are given in 10^9/ml (Fig.25).

Figure 25: picture of the photometer SDM6 (by Minitüb GmbH and Cryogenetics®) showing the data output. In the example the measurement of the milt concentration is 17,148*10^9 /ml.

One hundred and thirteen data of sperm concentration have been collected and the results were collected in the boxplot in Fig. 26 divided per month of sampling. Complete table for concentration data can be found in Appendix E.

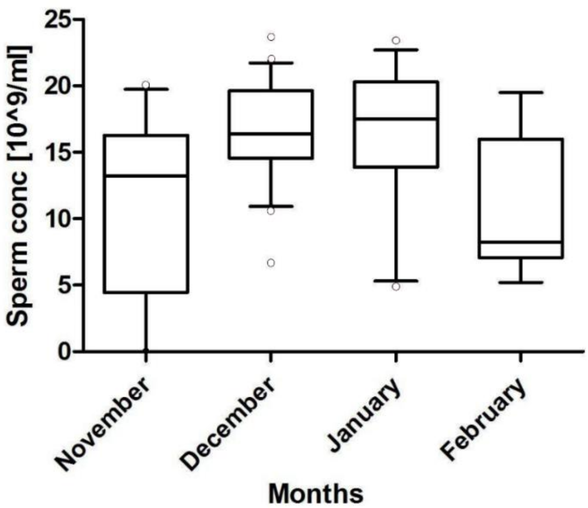

Figure 26: Boxplot displaying data from concentration assay divided per month of sampling. Higher values of the concentration are recorder in December and in January

Considering all the reproductive season, the sperm concentration in analyzed breeders resulted high, on average, with a mean of 14.95*10^9 of spermatozoa per ml. Calculated frequencies of the milt concentration results showed that the most individuals presented values between 12.5 and 22.5*10^9 sperm per ml (Figure 27; Zuccon *et al.*, in press).

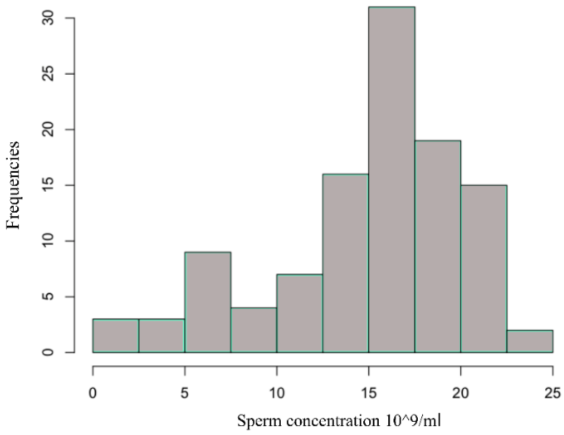

Figure 27: Barplot displaying data from concentration assay in terms of frequencies of the milt concentration. Most individuals have values between 12.5 and 22.5*10^9 sperm per ml with a mean of 14.95*10^9 of spermatozoa per ml.

4.4 Concentration during reproductive season

Nine males, three for each river basin, were stripped every week during the reproductive season. Complete table for concentration data can be found in Appendix F; the tendency graphic obtained from the continuous measurement are displayed in Fig. 28.

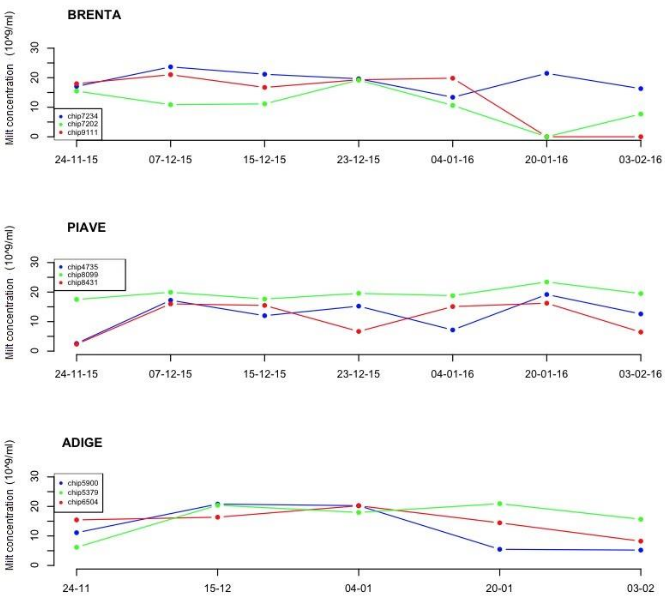

Figure 28: graphics displaying the milt concentration tendency of nine trout males during the reproductive season. On the *y* axes the milt concentration values expressed in 10^9/ml; on the *x* axes the collection data (gg-mm-yy). In the small box the legend that tells the chip, or barcode number, of every individual monitored.

One-way ANOVA two-tailed with Tukey's post test was performed using GraphPad Prism version 7 (GraphPad Software) in order to test if there was a statistically significant difference between animals belonging to different river basins. The results of the test was a p-value > 0.05 indicating that there is not a significant difference in seminal liquid concentrations between breeders coming from different basins.

An unpaired t test two-tailed with Welch's correction was performed using GraphPad Prism version 7 (GraphPad Software) in order to test differences between pure marble trout and hybrids. The correction was made because the standard deviations calculated between the two groups resulted different. The results of the test was a p-value > 0.05 indicating that there is not a significant difference in milt concentration between pure marble trout breeders and hybrids.

4.5 Artificial fertilization

4.5.1 Egg fertilization experiment

In the table in Appendix G are reported the total results of the egg fertilization experiment; in figure 29 an extract of the table.

DATE	FISH ID	RIVER	MOTILITY	CONCENTRATION 10^9/ml	MILT USED	BORN	DEAD
25-11-15	275	Brenta	2	20,00	Dil	19	281
25-11-15	275	Brenta	2	20,00	Non-dil	130	170
25-11-15	738	Brenta	3	14,53	Dil	53	247
25-11-15	738	Brenta	3	14,53	Non-dil	152	148

Figure 29: extract from the table in Appendix D reporting all the data from the egg fertilization experiment. In the first column the date of the artificial fertilization; in the second column the last four numbers of the male breeders barcode; in the third the river basin of origin; in the fourth the motility value; in the fifth the milt concentration in 10^9/ml; in the sixth the kind of milt used for the fertilization (Dil: diluted; Non-dil: non diluted); in the seventh the number of born fry and in the eight the number of dead eggs.

Only males with a milt concentration greater than $14*10^9$/ml and motility value of 2 or 3 were used for the fertilizations except for rare occasions, like at the end of the reproductive season. The choice of using males with higher values was due to the purpose of maximize the odds of eggs fertilization. This experiment was conducted on 20 fertilization in seven different days in the period from the 23rd of November 2015 until the 3rd of February 2016; a total of 12.000 eggs was fertilized. Results of the fertilization (hatched eggs *vs.* dead eggs) are displayed in the histogram 100% stacked columns (Fig.30). Diluted milt had a negative rate (2933 hatched eggs/ 3067 dead eggs) while the non-diluted milt had a positive rate (3798 hatched eggs/ 2202 dead eggs). Considering also the percentage, as shown in figure, the performance of the non-diluted milt led to better results.

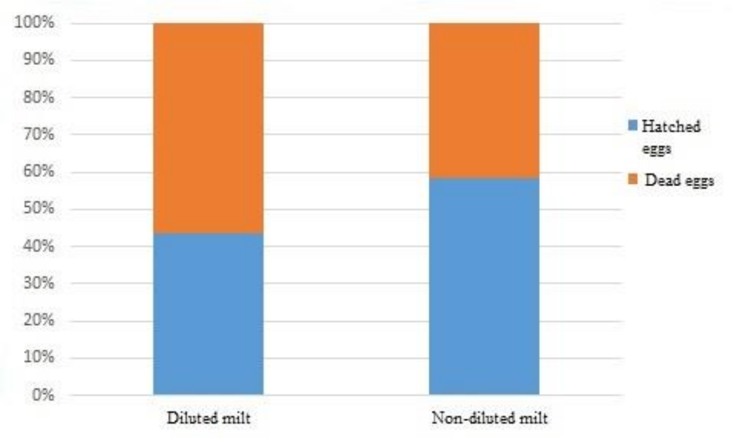

Figure 30: histogram 100% stacked columns displaying the results of the egg fertilization experiment. In the first column the results in terms of hatched and dead eggs for the fertilization with the diluted milt. In the secon column the results in terms of hatched and dead eggs for the fertilization with the non-diluted milt.

4.5.2 Statistical analyses for the egg fertilization experiment

For the Pearson's chi-squared test χ^2 (Pearson, 1900) with R Software (R Core Team, 2015) was created a matrix, named M, including the number of dead eggs and fry and born fishes using both the diluted and non-diluted milt. The results, as shown in Fig.31, was a p-value < $2.2*10^{-16}$. In order to accept the alternative hypothesis the p-value had to be minor than 0.05 so, for this experiment, the null hypothesis had to be be rejected.

```
> M
     [,1] [,2]
[1,] 3858 2442
[2,] 2978 3322
> chisq.test(M)

	Pearson's Chi-squared test with Yates' continuity correction

data:  M
X-squared = 247.07, df = 1, p-value < 2.2e-16
```

Figure 31: screenshot from R Software (R Core Team, 2015) showing the matrix (M) used in the Pearson's Chi-squared test and the results of the test. The value of X-squared is 247.07; df=1 indicate the degree of freedom followed by the p-value.

4.6 Cryopreservation

The milt of the breeders with the higher values of motility and sperm concentration were shipped successfully to Cryogenetics AS based in Hamar (Norway) that was responsible for quality assessment of samples after the expedition. They are still preserved inside a 47 liters dewar filled with liquid nitrogen and whenever the hatchery director will decide to use them, the company will ship the requested samples back to Centro Ittico Valdastico. Once in the hatchery operators will easily proceed with the artificial fertilization following a thawing protocol, given by the company, for the milt samples and then continuing with normal procedures. Fertilization with cryopreserved milt have a high yield, according to the company is the 80% in salmons, but results can be affected also by other factors, such as quality of the sperm before cryopreservation, egg quality and conditions during incubation. It must be remarked that this is a first experience at national level. Cryopreserved milt has been stored at Cryogenetics AS for future use. No cryopreserved milt was therefore used in this work.

5. Discussion

5.1. Mitochondrial haplotyping and LDH-C1 genotyping*

Combining mitochondrial and nuclear markers after a strict morphological characterization allowed the identification of 189 samples of putative pure marble trout breeders among the dataset of 229 breeders. Considering the maternal inheritance of mitochondrial DNA, since 100% of results showed marble haplotype, is possible to conclude that the genetic introgression was caused by males of brown trout. This asymmetrical hybridization can have many causes like ecological and ethological differences between the two species as studied in other Salmonids (Kitano *et al.*, 1994; Kanda *et al.*, 2002; Rubidge & Taylor, 2004; DeHaan *et al.*, 2010). In this taxon, however, hybridization is often unidirectional even though have been reported spatial and temporal variations in the patterns (Redenbach & Taylor, 2003; Baumsteiger *et al.*, 2005; Kozfkay *et al.*, 2007; Gunnell *et al.*, 2008; DeHaan *et al.*, 2010). In marble trout case the dynamics of this phenomenon are not deeply studied in Italy; only few studies regarding asymmetrical hybridization were conducted in the country like for the southern and northern pike (Gandolfi *et al.*, 2017) or in case of higly introgressed marble trout population in Piemonte region (Zerunian, 2003). A crucial role in this unidirectional hybridization can be ascribed to zootechnics activities. It's is possible to presume that in the past, in a period where hatcheries didn't take advantage of genetic analyses, this industry produced hybrids derived from marble trout females and brown trout males and released them in the rivers. A subset of 90 specimens derived by combined characterization was then submitted to microsatellite analyses.

5.2 Microsatellite analyses

5.2.1 Microsatellite panel on Centro Ittico di Valdastico (VI) marble trouts

The mean heterozygosity in groups values were low compared to heterozygosity levels in other teleost species often variable from 70% to 90% and, for conservation and management plans in hatcheries, this is a really important issue. Low heterozygosity observed in marble trout in Centro Ittico Valdastico could be attributed to inbreeding phenomenon that are common in hatcheries (Matusse *et al.*, 2016). Observing the bar plot from STRUCTURE 2.3.4 (Fig. 19) it is possible to notice that one individual in the Piave group showed genetic correspondence with specimens present in Adige group. The barcode of the PIT tag of that animal was checked and was discovered that the individual was indeed coming from Adige river basin, probably erroneously moved to the Piave tank. This event contributed to support the validity of the microsatellite panel tested. Since the Bayesian analyses showed a "population assignment" corresponding to the geographical origin of individuals, breeders were kept divided in three tanks in order to proceed with the fertilization and maintain the different genetic strains. Seeing as how the difference between these basins is not only geographical but also genetic is possible to speak about these three strains in terms of Management Units or MUs (Moritz, 1994; Palsbøll *et al.*, 1996; Stephenson, 1999; Reiss *et al.*, 2009) which is really important in conservation management plans (Zuccon *et al.*, in press).

5.2.2 Microsatellite panel on total dataset of trouts

Since the 12 microsatellite panel was resolved in distinguishing the river basin of origin of the 56 marble trout we decided to increase both the number of the microsatellite loci and the number of breeders tested, adding to the dataset also Mediterranean trouts individuals. The Belluno and Bassano del Grappa hatcheries claimed that their breeders came from Piave river basin and Brenta/Cismon river basin (see table I in *2.1* paragraph). Observing the bar plot from STRUCTURE 2.3.4 (Fig. 32) is possible to notice that breeders from Piave and Brenta/Cismon river basins have genetic similarities with the marble trouts of Centro Ittico di Valdastico (VI).

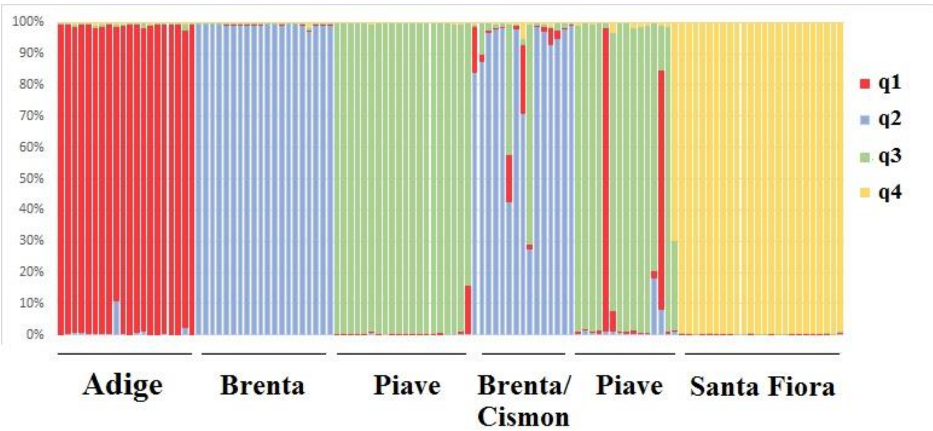

Figure 32: STRUCTURE software analyses on the whole microsatellite dataset. Clustering analysis of the entire dataset for estimated K = 3; q1, q2, q3 and q4 represent the q-values, expressed as probability values of each sample to belong to a specific cluster. On the *x* axe the river basin of origin of the trouts: Adige, Brenta and Piave river for the trouts from Centro Ittico di Valdastico (VI), Brenta/Cismon and Piave for trouts from another hatchery in Veneto that gave some marble trouts to Valdastico and Mediterranean trouts of Santa Fiora in Tuscany.

However, from the plot is evident that trouts from the Belluno and Bassano del Grappa hatcheries display genetic characteristics mixed between the three river basins. In particular three individuals from both Brenta/Cismon and Piave basins shows genetic similarities with

Piave and Adige MUs; one individual shows even a high percentage of genetic similarity with Mediterranean trouts from Santa Fiora (GR).

Since *S. cettii* trouts from Tuscany form a different cluster we can conclude that this microsatellite panel is resolved in distinguishing the two species and even in detecting hybrids. In fact individual 155a, coming from Belluno hatchery and assigned to the Piave MU, presents a high percentage of genetic introgression from Mediterranean trout. These evidences of genetic pollution are indicative of a management not attentive of the stocks in Belluno and Bassano del Grappa hatcheries on the contrary of what is done in Centro Ittico di Valdastico.

The FCA 3D analysis results showed a clear separation between marble trout breeders and Mediterranean trouts with one median individual between them (Fig. 22). In the marble trout group is possible to distinguish the Valdastico trouts in the three river basins of origin while breeders coming from hatcheries in Bassano del Grappa and Belluno cluster halfway between Piave and Brenta Valdastico trouts, as seen also with STRUCTURE analyses. In the FCA 2D graph for axes 1 and 2 (Fig. 23 a) is possible, again, to see separated the two species plus is evident that an individual, assigned to the Piave river from Belluno hatchery, is nearer to the group of Mediterranean trouts. Once checked the code of the animal it was confirmed that was the same breeder that presented a high percentage of *S. cettii* genotype spotted in STRUCTURE barplot.

Application of an additional bioinformatic index, supported the general view of separation between marble trout and Mediterranean trouts with values of Nei's distance higher than D=0,8. The Nei's genetic distance between Santa Fiora trouts and Piave trouts from Belluno is lower (D=0,667) probably because of the hybrid individual already cited in FCA and STRUCTURE analyses.

5.3 Motility and concentration assay

Since samples showed no significant evidence between river basins results were analysed based only on time. As shown in the plot in Fig. 24, most of the milt samples, 66.97%, showed a high sperm motility (between 80-100% of spermatozoa moving) during the four months of sampling. In November and in February the number of individuals that showed asthenozoospermia, from 0% (0 in the plot) to 20-40% (1 in the plot), were higher than in the center period of the reproductive season. The mean value for the motility assessed during four month sampling is 2,51 (corresponding to 50-70% of spermatozoa moving). It is interesting to notice that in the short term, between collection of samples and analyses, urine and feces contamination when present in low quantity did not affect the motility of sperm.

As shown in the plot in Fig.25, the higher values of the concentration were observed in December and in January, central months of this reproductive season while in November and in February the variability of the data were higher. This distribution is consistent with the reproductive physiology of the fish: production of milt in teleost increase until the reach of a peak in the middle of the reproductive period then decrease. Being able to detect the peak of the production in male breeders is important in order to plan the artificial reproduction in the successive seasons.

Breeders with a measure higher than 15 were always used, also if the motility was 2 or more, for artificial fertilization during the reproductive season. Oligozoospermia was found mostly in the first month of sampling, November, and, in minor proportion, in the last two months of the reproductive season. Likely for the motility assessment, contamination of urine and feces didn't affect the read of the photometer (Zuccon *et al.*, in press).

5.4 Concentration during reproductive season

In the group of nine males monitored during all the reproductive season, to observe the seasonal variability of the milt concentration, was not possible to distinguish a trend in order to identify a specific period in which the value was maximum for all the breeders. Being able to identify a time lapse of high milt concentration would be very convenient both because the fertilization rate could be higher and because this could allow operators to strip males only in the best moment of the reproductive season. Stripping is equivalent to stress fishes: sedation, manipulation and deep stripping can compromise the health of the animals and, in some cases, lead to their death. From the data collected is possible to observe that the time lapse in which is recorded a higher milt concentration, for almost all the marble trout males, is from the first days of December until the first days of January. It is believed that, if it was possible to continue the analyses after the beginning of February, could be observed a bell-shaped curve illustrating the end of milt production after the reproductive season. From the tendency graphic of the seasonality is possible to observe that males have a very long timescale of high milt concentration (greater than $15*109^9$ sperm/ml); this is indicative that they are not the limiting factor of a higher number of fertilization during the reproductive season. In effect, in hatcheries, the same male can be stripped more than once during reproductive period because his milt production is continuous and persist until the end of the season. For females is quite different because they produce and can lay eggs only once per year and they cannot be stripped again till the sequent reproductive season, the year after. Here in Fig. 33 is reported the graphic found in 3.4 paragraph with the add of lines indicating the time span in which female from the same basin were ready to produce eggs. First is possible to observe that female breeders from different basins have a different time lapse of reproductive period; for example, Brenta females were spawning during all the season, from the 23rd of November 2015 until the 3rd of February 2016, permitting a higher number of fertilization and production of offsprings for that basin.

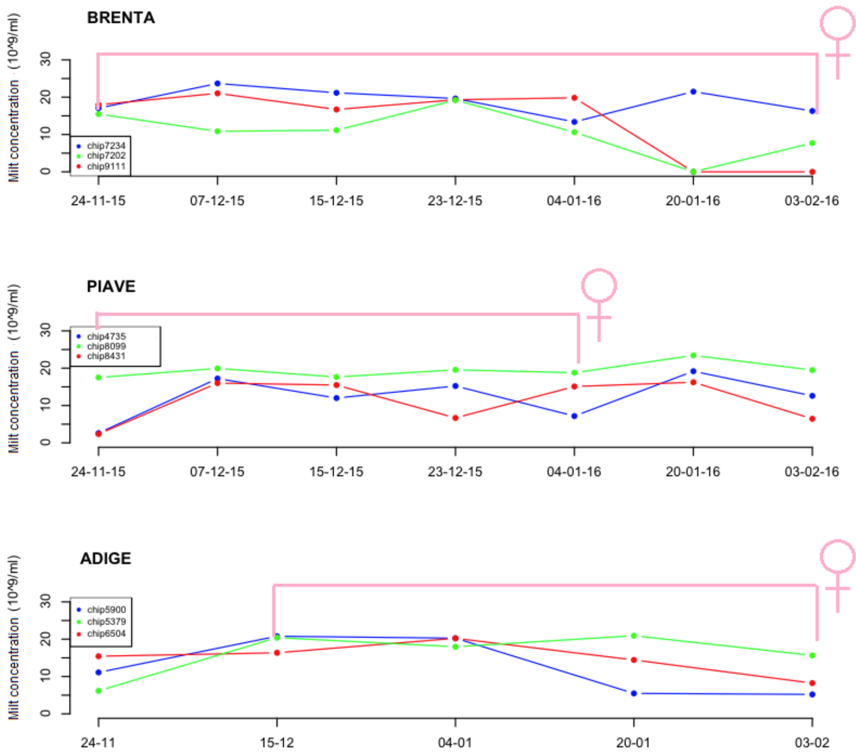

Figure 33: graphics displaying the milt concentration tendency of nine trout males during the reproductive season. On the *y* axes the milt concentration values expressed in 10^9/ml; on the *x* axes the collection data (gg-mm-yy). In the small box the legend that tells the chip, or barcode number, of every individual monitored. Pink brackets show the time span in which female from the basin were ready to hatch eggs.

From these graphs is possible to observe that females represent the real limiting factor in artificial fertilization in the hatchery: in fact, looking at the Piave basins graphic, despite in the last two days of analyses breeders showed a high milt concentration, optimal for fertilizations, no more females from the same basin were ready to hatch so it was not possible to strip males and use the milt. Regarding fishes belonging to Adige basin was observed a similar scenario: although since from the first days of stripping, in the late November, males had good milt concentration values, there were not detected females ready to hatch until the middle of December. Therefore, the effective reproductive season for Adige marble trout was postponed of about 20 days towards Brenta and Piave basins.

It is important to point out that in Centro Ittico di Valdastico the water in the tanks comes from a spring with a constant temperature of about 10,8-11°C, as opposed to what happens in nature; despite this we don't think it could affect the beginning of the reproductive season since these animals regulate mainly on the photoperiod (Bon *et al.*, 1999).

5.4 Egg fertilization experiment

Results of the Pearson's chi-squared test χ^2 (Pearson, 1900) on hatched and dead eggs showed a highly significant difference between the two different treatment (diluted *vs.* non-diluted milt). In particular has been observed a significant decrease of hatched eggs rate with the use of diluted milt. It is really important to point out that eggs, once fertilized and placed in the incubators, can die for causes that fall outside of the missed fertilization. Fungal infections, low quality of the eggs, due to a too young, too old or stressed female breeder (Simčič *et al.*, 2005; Lucarda *et. al*., 2007), and wrong movement made by the operators during the positioning of boxes in the incubator can all lead to the dead of the eggs. Fertilized eggs are really sensitive in the first days after they are put in the incubator that small collision, too much light or subsequent manipulation can be fatal in the development of the fry. It is also important to highlight that, for some experiment, eggs were mixed for mistake by the operators during the transfer of the boxes in the vertical incubator (Fig.34) so the final count was not entirely precise.

Figure 34: picture showing an operator checking for eyed eggs in the boxes in the vertical incubator. Note that the white plastic boxes are divided in four by two pieces of plastic. These X shaped pieces are not fixed on the box so eggs can roll from a space to another if it's not payed much attention during the transfer operation.

Saprolegniaceae fungal infections are the most frequent causes of death for eggs in hatcheries; in order to reduce the risk of epidemic between the eggs in incubation is important to remove infected or dead eggs, before the eyed eggs stage, by aspiration or with small tweezers paying attention to the near vital eggs (Fig.35); however this operation is possible only in horizontal incubators.

Figure 35: removal of dead eggs with tweezers. The dead ones are easily recognizable because of their white opaque color. Vital eggs are light orange and opaque, inside it’s possible to see the eye of the embyo.

The sanitary monitoring on the fish should be continuous until the fry is 4-6 cm in length as reported in Gatti and Barberi “La protezione sanitaria in troticoltura”.

6. Conclusions

As recently demonstrated by Chiesa *et al.* (2016), a combined approach based on single locus and multilocus fingerprinting is particularly useful in conservation and management plans of threatened fish populations. In fact, being able to previously select fish morphologically and then genetically shrink the stock of wild breeders to highly selected strains is important to guarantee the breed of pure animals to be carried out in ichthyogenic centers. In the case of marble trout *Salmo marmoratus* there is an increasing need for new and more incisive plans and restocking activities since, despite the efforts put in recent conservation plans, the Italian IUCN committee has raised its risk status from LC (Least Concern) to CR (Critically Endangered) over the last few years (Rondinini *et al.*, 2013).

Besides several environmental threats, an important role in this decline is also due to the hybridization with brown trout S. trutta that leads to genetic introgression decreasing the number of pure marble trout breeders and, consequently, offspring in the wild. Morphological identification of hybrids is trivial, especially if examined animals do not belong to the first generations produced. For this reason, a molecular approach is strictly recommended when the putative marble trouts are transferred in hatcheries.

In this work, the combined approach based on mitochondrial SNPs multiplex detection and nuclear microsatellite analyses demonstrated a clear differentiation among populations to be submitted to artificial insemination. In particular, microsatellite analyses on the whole trout dataset were able to confirm the presence of three different clusters corresponding with the river of origin of the marble trout breeders and to distinguish them from a fourth cluster represented by Mediterranean trouts. The multi-marker approach showed a clear resolution also for detection of low level cryptic hybridization. Moreover STRUCTURE analyses showed also a genetic pollution phenomenon occurred both in the hatcheries of Belluno and Bassano del Grappa.

In order to correctly manage an endangered species it is really important to maintain separated the populations that show genetic differences and treat them as different Management Units (MUs). For this reason, having a high number of molecular markers can help in the identification both of some hybrids and of the breeders that show a higher allelic richness and genetic variability in order to advance the use of their gametes during the reproductive season.

Highly selected marble trouts were subsequently submitted to an innovative approach based on new technologies in the field of reproductive biology. In particular, the use of a photometer dedicated to measuring the sperms concentration of milt belonging to different fish species has demonstrated his usefulness in artificial reproduction. A simple dark-field microscope and the SDM6 are time and space-savings tools. A trained technician can make evaluations, for the stripped males, in less than one hour while the operators in the hatchery are stripping the females and preparing the eggs for the fertilizations. The choice of the males with higher concentration of sperm and a good motility is important in order to obtain higher fertilization rate during the season, especially for the endangered species, whose offsprings are going to be released in rivers. Frequently we can observe a lack of synchronization in hatcheries between the availability of milt and the production of eggs. Detecting the best time for stripping males, both in terms of motility and concentration of sperms, is crucial when the collection of milt is paired with cryopreservation.

Collecting and storing the milt in liquid nitrogen has several advantages: i) it acts as a genetic backup of the males present in hatcheries; ii) it represents a chance, for other conservation-based ichthyogenic centers, to increase the genetic variation of stock coming from the same river basin (Martínez-Páramo et al., 2010); iii) it permits to hatcheries to stripe males and then release them in nature avoiding sanitary problems related to the fighting for mating choice during the reproductive season (Zuccon *et al.*, in press).

The combination of molecular analyses and new technologies have demonstrated to be two important tools useful for more efficient management plans for conservation of endangered salmonid species.

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Appendix A

Breeders of S. marmoratus collected from the hatchery Centro Ittico Valdastico in the Veneto region, North Italy.

Trouts from Adige river transferred in hatchery in 2010

SAMPLE NUMBER	SEX	BARCODE	LENGHT cm	WEIGHT kg	D-LOOP	LDH-C1*
1	M	968000004730958	47	1,29	MA	ME
2	M	968000004568954	51	1,605	MA	ME
3	M	968000004746968	40	0,755	MA	ME
4	M	968000004273144	53	2,065	MA	ME
5	M	968000004566595	50,5	1,83	MA	HET
6	M	968000004736315	45	1,03	MA	ME
7	M	968000004735379	48	1,275	MA	ME
8	M	968000004749072	41	870	MA	ME
9	M	968000004567116	47	1,2	MA	ME
10	M	968000004706880	47	1,345	MA	ME
11	M	968000004729696	56	2,395	MA	ME
12	F	968000004568889	43,5	1,14	MA	ME
13	F	968000004734204	42,5	1,075	MA	HET
14	F	968000004767131	50,5	1,5	MA	ME
15	F	968000004567143	44	1,095	MA	ME
16	F	968000004756941	51	1,73	MA	ME
17	F	968000004739222	47	1,385	MA	ME
18	M	968000004744913	45	1,045	MA	ME
19	M	968000004565900	49	1,52	MA	HET
20	M	968000004706504	48	1,24	MA	ME
60	M	968000004747805	57,5	1,005	MA	ME

Trouts from Brenta river transferred in hatchery in 2009

SAMPLE NUMBER	SEX	BARCODE	LENGHT cm	WEIGHT kg	D-LOOP	LDH-C1*
21	M	968000004267483	48	2	MA	ME
22	M	968000004737468	44	1	MA	ME
23	M	968000004569214	47	1	MA	ME
24	M	968000004745592	49	1	MA	ME
25	M	968000004748237	48	2	MA	ME
26	M	968000004740155	44	1	MA	ME
27	M	968000004735392	38	1	MA	ME
28	M	968000004747447	49	1	MA	ME
29	M	968000004571902	51	2	MA	ME
30	M	968000004830738	51	2	MA	ME
31	M	968000004762916	46	1	MA	ME
32	M	968000004748438	47	1	MA	ME
33	F	968000004725604	45	1	MA	ME
34	F	968000004705547	47	2	MA	ME
35	F	968000004747167	43	1	MA	ME
36	F	968000004568996	44	1	MA	ME
37	F	968000004748093	41	1	MA	ME
38	F	968000004729583	44	1	MA	ME
39	F	968000004741141	39	1	MA	ME
40	F	968000004269688	44	1	MA	ME

Trouts from Piave river transferred in hatchery in 2008

SAMPLE NUMBER	SEX	BARCODE	LENGHT cm	WEIGHT kg	D-LOOP	LDH-C1*
41	M	968000004569125	55	2	MA	ME
42	F	968000004707174	47	1	MA	ME
43	M	968000004729678	49	1	MA	ME
44	M	698000004727888	57	2	MA	ME
45	M	968000004749944	50	2	MA	ME
46	M	968000004728308	49	1	MA	ME
47	M	968000004567749	51	2	MA	ME
48	M	968000004730384	48	1	MA	ME
49	F?	968000004738281	47	1	MA	ME
50	F	968000004271145	44	1	MA	ME
51	F	968000004748320	41	1	MA	ME
52	F	968000004741962	49	1	MA	ME
53	F	968000004747405	44	1	MA	ME
54	F	968000004727737	45	1	MA	ME
55	F	968000004570004	48	1	MA	ME
56	F	968000004739882	44	1	MA	HET
57	F	968000004747664	60	3	MA	ME
58	M	968000004572572	48	1	MA	ME
59	M	968000004567387	52	2	MA	ME

Trouts from Associazione Bacino Acque Fiume Brenta transferred in hatchery in 2014

SAMPLE NUMBER	CODE	LENGHT cm	WEIGHT kg	D-LOOP	LDH-C1*
1	1910251	37	0,675	MA	ME
2	1971917	37	0,525	MA	ETER
3	2036581	45	1,11	MA	ME
4	1936462	42	0,775	MA	ME
5	1841077	35	0,675	MA	ME
6	2019401	46	1,195	MA	ME
7	1866632	43	0,855	MA	ME
8	1819445	45	1,025	MA	ME
9	1937888	34	0,38	MA	ETER
10	1948881	34	0,36	MA	ETER
11	1990803	36	0,52	MA	ETER
12	1959111	44	1,05	MA	ETER
13	1947234	42	1,055	MA	ETER
14	1725244	32	0,395	MA	ME
15	1900413	40	0,84	MA	ETER
16	1825241	41	0,685	MA	ME
17	1924464	42	0,935	MA	ME
18	1949263	42	0,9	MA	ME
19	1752922	43	0,985	MA	ETER
20	1991222	40	0,695	MA	ME
21	2038294	42	0,89	MA	ME
22	1974549	46	1,235	MA	ETER
23	1967632	40	0,765	MA	ME
24	1944455	40	0,7	MA	ME
25	1945699	45	0,96	MA	ME
26	1910354	34	0,555	MA	ME
27	1931664	56	2,5	MA	ME
28	1954799	47	1,235	MA	ME
29	2017761	40	0,705	MA	ME
30	2037733	41	0,8	MA	ME
31	19522688	40	0,71	MA	ME
32	1992492	35	0,6	MA	ME
33	1720248	39	0,69	MA	ME
34	1797981	45	1,5	MA	ETER
35	2038335	50	1,325	MA	ME
36	2016794	44	0,97	MA	ME
37	1913666	41	0,905	MA	ETER
38	1976178	35	0,53	MA	ETER
39	1951130	35	0,535	MA	ME
40	1945926	43	0,875	MA	ME
41	1810615	34	0,445	MA	ETER
42	1757262	43	0,875	MA	ME
43	1960089	41	0,84	MA	ME
44	2011792	46	1,41	MA	ME
45	2616146	40	0,725	MA	ME
46	1888482	53	1,825	MA	ME
47	1967202	40	0,52	MA	ME
48	1908757	39	0,775	MA	ETER
49	1819454	34	0,455	MA	ETER
50	1905158	40	?	MA	ME
51	1443702	45	1,025	MA	ME

52	1757852	47	1,1	MA	ME
53	2015119	39	0,7	MA	ETER
54	1815207	36	0,49	MA	ME
55	1807966	52	1,56	MA	ME
56	1951741	56	2,27	MA	ME
57	1955550	49	1,335	MA	ME
58	1805424	45	1,04	MA	ME
59	1952870	42	0,87	MA	ETER
60	1757076	45	1,035	MA	ME
61	1820708	44	0,97	MA	ETER
62	2017644	36	0,525	MA	ME
63	1954308	34	0,445	MA	ME
64	1935300	36	0,54	MA	ME
65	1876275	51	1,6	MA	ME
66	1936050	43	0,825	MA	ME
67	1828071	45	1,235	MA	ETER
68	1889656	45	1,07	MA	ME
69	1912164	42	0,905	MA	ETER
70	2014442	46	0,96	MA	ETER
71	1955563	35	0,49	MA	ME
72	1909081	39	0,595	MA	ETER
73	1948803	41	0,785	MA	ME
74	1931470	33	0,49	MA	ETER
75	1778271	33	0,46	MA	ETER
76	1910226	48	1,175	MA	ME
77	1930561	41	0,895	MA	ME
78	1851063	43	0,84	MA	ME
79	1821695	37	0,73	MA	ME
80	1781363	33	0,405	MA	ME
81	1916523	39	0,818	MA	ETER
82	1766616	33	0,39	MA	ME
83	1935257	38	0,66	MA	ME
84	2015624	36	0,565	MA	ME

Trouts from Piave river hatched in a hatchery in Belluno and transferred in hatchery in 2014

SAMPLE NUMBER	CODE	LENGHT cm	WEIGHT kg	D-LOOP	LDH-C1*
85	1825414	57	0,625	MA	ME
86	1955725	43	0,795	MA	ME
87	1945756	48	1,34	MA	ME
88	1950078	54	1,8	MA	ME
89	1950910	39	0,66	MA	ME
90	2037561	55	1,88	MA	ME
91	1915335	47	1,41	MA	ME
92	1916583	48	1,395	MA	ME
93	1908492	47	1,1	MA	ME
94	1889766	50	1,445	MA	ME
95	1907291	38	0,665	MA	ME
96	1825868	42	0,85	MA	ME
97	1908492	44	0,955	MA	ME
98	1811447	48	1,59	MA	ME
99	1990801	47	1,21	MA	ME
100	1843876	51	1,575	MA	ME
101	1816235	53	1,79	MA	ETER
102	1913269	44	0,9	MA	ATL
103	1888815	37	0,72	MA	ME
104	1971694	52	1,83	MA	ME
105	1889295	50	1,56	MA	ME
106	1946858	47	1,31	MA	ME
107	1867477	50	1,428	MA	ME
108	1977368	48	1,425	MA	ME
109	1907036	53	1,63	MA	ME
110	1951134	47	1,29	MA	ME
111	1890678	41	0,55	MA	ME
112	1890484	45	1,085	MA	ATL
113	1888431	52	1,625	MA	ME
114	2013281	37	0,555	MA	ME
115	1811683	51	1,555	MA	ME
116	1975730	54	1,32	MA	ME
117	1972028	59	2,285	MA	ME
118	1991478	46	1,115	MA	ME
119	1950102	48	1,64	MA	ME
120	1992057	43	1,01	MA	ME
121	2038133	46	1,08	MA	ME
122	1971105	45	1,145	MA	ME
123	1922674	44	1	MA	ME
124	1857300	49	1,395	MA	ME
125	1971603	44	1,035	MA	ME
126	1825763	46	1,295	MA	ME
127	1826384	44	1,143	MA	ME

128	2016077	52	1,47	MA	ME
129	1856077	53	1,7	MA	ME
130	1912067	48	1,395	MA	ME
131	1868099	44	0,935	MA	ETER
132	1911249	51	1,815	MA	ME
133	1974237	41	1	MA	ME
134	1929996	47	1,8	MA	ME
135	1953878	45	1,14	MA	ME
136	1953197	52	1,795	MA	ETER
137	1757775	50	1,71	MA	ETER
138	1968444	44	1,01	MA	ETER
139	1932780	46	1,09	MA	ME
140	1934735	47	1,295	MA	ETER
141	1961311	44	0,985	MA	ME
142	1919466	47	1,39	MA	ME
143	1966315	51	1,755	MA	ETER
144	1959843	51	1	MA	ETER
145	1818898	41	0,777	MA	ETER
146	1889618	52	1,13	MA	ME
147	1973898	47	1,2	MA	ME
148	1961954	43	1,075	MA	ME
149	1990392	52	1,79	MA	ME
150	1959449	50	1,?50	MA	ME
151	2015056	52	1,65	MA	ME
152	1908064	49	1,37	MA	ME
153	1909610	50	1,52	MA	ME
154	1961717	52	1,83	MA	ME
155	1888174	45	1,19	MA	ME
156	1973055	56	2,9	MA	ME
157	1768571	44	1,09	MA	ME
158	2038205	45	1,1	MA	ME
159	1812568	51	1,87	MA	ME
160	1889801	43	0,95	MA	ME
161	1888785	43	0,835	MA	ME
162	1955741	39	0,735	MA	ME
163	1976864	47	1,28	MA	ME
164	1962136	40	0,965	MA	ME
165	2018053	48	1,33	MA	ME
166	1848122	47	1,485	MA	ME
167	1810431	48	1,33	MA	ME
168	1960518	46	1,34	MA	ME
169	1968843	40	0,76	MA	ME

Appendix B

Microsatellites previously tested for *Salmo salar* and *Salmo marmoratus* and present in literature. Every table contains the data of a single panel and relative multiplex.

G5 (or Any5dye?)	**Panel I Trout**	**Size range**	**Final Conc (uM)**
FAM	BHMS349	100 - 135	0,044
ATTO550 or NED	SSaD85	150-195	0,019
FAM	SSaD58	175 - 250	0,064
FAM	STR2	300 - 400	0,027
YakYel	BHMS330	70 - 135	0,043
YakYel	BHMS429	175 - 230	0,019
NED (or ATTO550)	Tap2B	270 - 340	0,025
PET	SSa197	110 - 170	0,032
PET	SSaD157	237 - 355	0,056

G5 (or Any5dye?)	**Panel II Trout**	**Size range**	**Final Conc (uM)**
VIC	SSaD190	120 – 165	0,016
NED	SSaD170	135 - 200	0,012
PET	SSa85	95 - 135	0,010

G5 (or Any5dye?)	**Panel IIb Trout**	**Size range**	**Final Conc (uM)**
FAM	Str73INRA	120 - 150	0,018
FAM	SSa171	215 – 255	0,010
VIC	SSaD190	120 – 165	0,016
NED	SSaD170	135 - 200	0,012
PET	SSa85	95 - 135	0,010
FAM	Mst60	60 - 111	0,030
YakYel	Sfo8	216 - 318	0,030
YakYel	SfoC79	100 - 104	0,020

Any5dye	**Panel III Trout**	**Size range**	**Final Conc (uM)**
ATTO550	BHMS117B	102-112	0,040
ATTO565	BHMS269	90-150	0,040
YakYel	BHMS278	107-113	0,040
ATTO550	BHMS360	200-209	0,040
ATTO550	BHMS377	130-173	0,040
FAM	BHMS389	167-179	0,040
ATTO565	CL15589	158-168	0,040
YakYel	OMM1121/i	194-267	0,040
ATTO565	Ssa64/ii	212-242	0,040

Appendix C

Allelic richness per locus

BHMS330	AdV	BrV	PiV
(N)	20	20	20
90	0.6500	0.8500	0.8250
92	0.0500	0.0000	0.1500
94	0.0250	0.0000	0.0000
96	0.2250	0.0250	0.0250
102	0.0250	0.1000	0.0000
106	0.0000	0.0250	0.0000
110	0.0250	0.0000	0.0000
H exp.	0.5225	0.2662	0.2963
H n.b.	0.5359	0.2731	0.3038
H obs.	0.6500	0.3000	0.1500

BHMS349	AdV	BrV	PiV
(N)	20	20	20
88	0.0000	0.0000	0.0250
90	0.0000	0.0250	0.0250
98	0.0000	0.2750	0.3750
106	0.0000	0.0250	0.0000
108	0.0000	0.2000	0.0000
116	0.0750	0.0250	0.0000
118	0.0500	0.0000	0.0250
122	0.5500	0.0000	0.0250
124	0.0250	0.0000	0.0750
128	0.1000	0.0000	0.0000
146	0.0000	0.0250	0.0500
148	0.1000	0.3750	0.3500
152	0.0000	0.0500	0.0500
999	0.1000	0.0000	0.0000
H exp.	0.6587	0.7388	0.7238
H n.b.	0.6756	0.7577	0.7423
H obs.	0.4500	0.7000	0.6500

BHMS429	AdV	BrV	PiV
(N)	20	20	20
177	0.0250	0.0000	0.0000
191	0.0000	0.0000	0.1750
195	0.6000	0.5250	0.7500
201	0.2000	0.4500	0.0250
205	0.1750	0.0000	0.0000

213	0.0000	0.0000	0.0500
217	0.0000	0.0250	0.0000
H exp.	0.5687	0.5213	0.4038
H n.b.	0.5833	0.5346	0.4141
H obs.	0.6000	0.6500	0.4000

SSaD85	AdV	BrV	PiV
(N)	20	20	20
148	0.0000	0.0250	0.0000
156	0.0250	0.0000	0.0000
176	0.0000	0.0250	0.0000
182	0.0000	0.0000	0.6750
186	0.0250	0.0750	0.0000
188	0.0250	0.0000	0.0000
190	0.0000	0.0250	0.0000
192	0.2750	0.0000	0.0000
196	0.0750	0.0000	0.0750
200	0.0000	0.1750	0.0000
204	0.0000	0.0250	0.0250
206	0.0000	0.0750	0.0000
208	0.0000	0.3500	0.0000
210	0.0500	0.0000	0.0000
212	0.1000	0.0000	0.0750
214	0.0000	0.0000	0.1500
220	0.1250	0.0000	0.0000
224	0.1250	0.0500	0.0000
228	0.0750	0.0000	0.0000
232	0.1000	0.0000	0.0000
234	0.0000	0.1750	0.0000
H exp.	0.8575	0.8000	0.5100
H n.b.	0.8795	0.8205	0.5231
H obs.	0.8500	0.7000	0.6000
STR-2	AdV	BrV	PiV
(N)	20	20	20
322	0.1500	0.0000	0.0000
324	0.0250	0.0000	0.0000
326	0.0250	0.1250	0.0000
330	0.0500	0.0000	0.0000
334	0.0250	0.0000	0.0500
340	0.0000	0.0250	0.0000
342	0.0000	0.0000	0.1250
344	0.0000	0.2250	0.0750
346	0.0500	0.0750	0.0500
350	0.0250	0.0000	0.0250

352	0.1000	0.1500	0.0000
356	0.0750	0.0000	0.0500
360	0.3250	0.0500	0.5250
372	0.0000	0.2000	0.0000
999	0.1500	0.1500	0.1000
H exp.	0.8263	0.8400	0.6850
H n.b.	0.8474	0.8615	0.7026
H obs.	0.6500	0.6000	0.4500

Ssa197	AdV	BrV	PiV
(N)	20	20	20
131	0.0000	0.0000	0.0250
137	0.0000	0.0000	0.2000
147	0.0000	0.1750	0.0000
157	0.0500	0.0000	0.1000
161	0.0000	0.0250	0.0000
165	0.0000	0.2750	0.0000
177	0.1250	0.2250	0.2750
179	0.0500	0.1250	0.0500
181	0.0000	0.1250	0.0750
183	0.0000	0.0000	0.0250
185	0.1750	0.0000	0.2500
189	0.0500	0.0250	0.0000
193	0.2000	0.0000	0.0000
197	0.1750	0.0000	0.0000
205	0.0500	0.0000	0.0000
213	0.0250	0.0250	0.0000
215	0.0250	0.0000	0.0000
225	0.0250	0.0000	0.0000
999	0.0500	0.0000	0.0000
H exp.	0.8688	0.8100	0.8025
H n.b.	0.8910	0.8308	0.8231
H obs.	0.9000	10.000	0.9000

Tap2B	AdV	BrV	PiV
(N)	20	20	20
271	0.0000	0.0500	0.0000
305	0.0250	0.0000	0.0000
313	0.4000	0.1000	0.8000
321	0.5250	0.8500	0.2000
999	0.0500	0.0000	0.0000
H exp.	0.5613	0.2650	0.3200

H n.b.	0.5756	0.2718	0.3282
H obs.	0.2500	0.2000	0.3000

Mst60	AdV	BrV	PiV
(N)	20	20	20
94	0.9000	0.7750	10.000
98	0.0000	0.2250	0.0000
999	0.1000	0.0000	0.0000
H exp.	0.1800	0.3487	0.0000
H n.b.	0.1846	0.3577	0.0000
H obs.	0.0000	0.3500	0.0000

Sfo8	AdV	BrV	PiV
(N)	20	20	20
194	0.0750	0.0000	0.0000
196	0.0000	0.0000	0.4000
200	0.0000	0.5000	0.3000
202	0.3500	0.4500	0.1250
204	0.4000	0.0000	0.0500
226	0.0250	0.0000	0.0000
254	0.0000	0.0000	0.0250
999	0.1500	0.0500	0.1000
H exp.	0.6887	0.5450	0.7212
H n.b.	0.7064	0.5590	0.7397
H obs.	0.3500	0.6000	0.6000

SfoC79	AdV	BrV	PiV
(N)	20	20	20
103	0.0250	0.0000	0.0000
105	0.0000	0.0000	0.0250
123	0.8750	0.9750	0.9500
133	0.0000	0.0250	0.0250
999	0.1000	0.0000	0.0000
H exp.	0.2237	0.0487	0.0962
H n.b.	0.2295	0.0500	0.0987
H obs.	0.0500	0.0500	0.1000

Str73INR	AdV	BrV	PiV
(N)	20	20	20
138	0.0000	0.4000	0.0750
144	0.0500	0.0250	0.0000

146	0.0000	0.2500	0.0000
150	0.2000	0.1250	0.2750
154	0.1000	0.0000	0.0000
156	0.0500	0.0000	0.0000
160	0.1500	0.0750	0.6250
162	0.0000	0.0000	0.0250
164	0.3000	0.1250	0.0000
999	0.1500	0.0000	0.0000
H exp.	0.8100	0.7400	0.5275
H n.b.	0.8308	0.7590	0.5410
H obs.	0.5000	0.8500	0.5000

BHMS360	AdV	BrV	PiV
(N)	20	20	20
163	0.1250	0.0000	0.0500
174	0.0000	0.0000	0.2750
207	0.0000	0.0000	0.1500
209	0.1250	0.0000	0.0000
211	0.0000	0.5000	0.0000
215	0.0500	0.0000	0.0000
219	0.0750	0.3250	0.0000
221	0.2500	0.0000	0.0000
223	0.0000	0.1000	0.0000
227	0.0000	0.0250	0.0000
231	0.1250	0.0000	0.0250
237	0.0000	0.0250	0.0000
239	0.0000	0.0250	0.0750
241	0.0250	0.0000	0.0500
243	0.0000	0.0000	0.1750
259	0.0000	0.0000	0.1750
305	0.1250	0.0000	0.0000
311	0.1000	0.0000	0.0000
321	0.0000	0.0000	0.0250
H exp.	0.8562	0.6325	0.8287
H n.b.	0.8782	0.6487	0.8500
H obs.	0.9000	0.6000	10.000

BHMS389	AdV	BrV	PiV
(N)	20	20	20
179	0.1250	0.3000	0.0500
191	0.0000	0.0250	0.0000
199	0.0000	0.0000	0.1000
249	0.0000	0.2750	0.0000

255	0.0250	0.0000	0.0500
257	0.0250	0.0000	0.3000
259	0.3000	0.0000	0.0000
261	0.0250	0.0000	0.0000
275	0.0250	0.0000	0.0000
277	0.0750	0.0000	0.0000
281	0.1750	0.0000	0.1000
283	0.0250	0.3750	0.0000
285	0.0500	0.0250	0.1250
287	0.0750	0.0000	0.2750
305	0.0250	0.0000	0.0000
999	0.0500	0.0000	0.0000
H exp.	0.8438	0.6925	0.7937
H n.b.	0.8654	0.7103	0.8141
H obs.	0.7000	0.7000	10.000

CL15589	AdV	BrV	PiV
(N)	20	20	20
207	0.0000	0.0000	0.2750
209	0.1500	0.1250	0.0000
211	0.0000	0.3500	0.0000
215	0.0500	0.0000	0.0000
217	0.0250	0.1000	0.0000
219	0.3500	0.2250	0.0000
223	0.0750	0.0500	0.0000
225	0.0000	0.0500	0.0000
227	0.0000	0.0250	0.0000
231	0.1250	0.0000	0.0000
237	0.0000	0.0500	0.0500
239	0.0250	0.0000	0.0750
241	0.0000	0.0000	0.3000
257	0.0000	0.0000	0.2000
301	0.0250	0.0000	0.0000
313	0.0750	0.0250	0.0500
999	0.1000	0.0000	0.0500
H exp.	0.8138	0.7925	0.7812
H n.b.	0.8346	0.8128	0.8013
H obs.	0.6000	0.6500	0.4000

OMM1121i	AdV	BrV	PiV
(N)	20	20	20
203	0.0000	0.1500	0.0000
222	0.0250	0.0000	0.0000

223	0.1250	0.0000	0.0000
225	0.0250	0.0000	0.0750
228	0.0000	0.0250	0.0000
230	0.1500	0.0000	0.1000
236	0.4000	0.5000	0.3500
241	0.0000	0.0000	0.3250
243	0.0000	0.0250	0.0000
259	0.0000	0.0000	0.0500
261	0.0000	0.0250	0.0250
312	0.0500	0.0000	0.0000
318	0.0250	0.0000	0.0000
327	0.0000	0.0500	0.0750
330	0.0000	0.1250	0.0000
334	0.1500	0.0750	0.0000
342	0.0000	0.0250	0.0000
999	0.0500	0.0000	0.0000
H exp.	0.7725	0.7012	0.7475
H n.b.	0.7923	0.7192	0.7667
H obs.	0.9000	10.000	10.000

AdV= Adige Valdastico

BrV= Brenta Valdastico

PiV= Piave Valdastico

(N)= number of individuals

In the first column the locus and the allele observed.

H exp.= heterozygosity calculed with bias

H n.b.= heterozygosity calculed without bias (Nei 1978)

H obs. = heterozygosity observed

Appendix D

Allelic richness per locus

BHMS330	AdV	BrV	PiV	BrBG	PiB	SF
(N)	20	20	20	15	15	24
76	0	0	0	0	0	0,0417
82	0	0	0	0	0,2333	0,0625
90	0,65	0,85	0,825	0,7667	0,5667	0
92	0,05	0	0,15	0	0,1333	0
94	0,025	0	0	0,0667	0	0,0833
96	0,225	0,025	0,025	0	0	0,0833
101	0	0	0	0,0333	0	0,625
102	0,025	0,1	0	0	0	0
104	0	0	0	0,0667	0	0,1042
106	0	0,025	0	0,0667	0	0
110	0,025	0	0	0	0	0
114	0	0	0	0	0,0667	0
H exp.	0,5225	0,2662	0,2963	0,3978	0,6022	0,579
H n.b.	0,5359	0,2731	0,3038	0,4115	0,623	0,5913
H obs.	0,65	0,3	0,15	0,4	0,7333	0,4583

BHMS349	AdV	BrV	PiV	BrBG	PiB	SF
(N)	20	20	20	15	15	24
88	0	0	0,025	0	0	0,9375
90	0	0,025	0,025	0	0	0
98	0	0,275	0,375	0	0,0667	0
100	0	0	0	0,0667	0	0
106	0	0,025	0	0	0	0,0208
108	0	0,2	0	0,2	0	0
112	0	0	0	0	0,0333	0
114	0	0	0	0	0,1333	0
116	0,075	0,025	0	0	0	0
118	0,05	0	0,025	0	0,2	0
120	0	0	0	0,0667	0	0
122	0,55	0	0,025	0,0667	0,0333	0
124	0,025	0	0,075	0	0,2	0
128	0,1	0	0	0	0,0333	0
138	0	0	0	0,0333	0	0
144	0	0	0	0,1	0	0
146	0	0,025	0,05	0,1	0	0
148	0,1	0,375	0,35	0	0	0,0417
150	0	0	0	0,0667	0	0
152	0	0,05	0,05	0,1667	0,3	0
195	0	0	0	0,0333	0	0
201	0	0	0	0,0333	0	0
999	0,1	0	0	0,0667	0	0
H exp.	0,6587	0,7388	0,7238	0,8867	0,8044	0,1189
H n.b.	0,6756	0,7577	0,7423	0,9172	0,8322	0,1215
H obs.	0,45	0,7	0,65	0,5333	0,8	0,125

BHMS429	AdV	BrV	PiV	BrBG	PiB	SF
(N)	20	20	20	15	15	24
177	0,025	0	0	0,2	0,1	0
181	0	0	0	0,0333	0,1333	0
185	0	0	0	0,0667	0,1	0
189	0	0	0	0,1333	0	0
191	0	0	0,175	0	0,1	0,0625
195	0,6	0,525	0,75	0,4	0,4	0
201	0,2	0,45	0,025	0,1	0,0667	0,6042
205	0,175	0	0	0,0667	0	0
209	0	0	0	0	0	0,1042
213	0	0	0,05	0	0	0
217	0	0,025	0	0	0,0333	0
221	0	0	0	0	0	0,1875
999	0	0	0	0	0,0667	0,0417
H exp.	0,5687	0,5213	0,4038	0,7622	0,7822	0,5833
H n.b.	0,5833	0,5346	0,4141	0,7885	0,8092	0,5957
H obs.	0,6	0,65	0,4	0,6	0,4	0,625

SSaD85	AdV	BrV	PiV	BrBG	PiB	SF
(N)	20	20	20	15	15	24
148	0	0,025	0	0	0	0
156	0,025	0	0	0	0	0
170	0	0	0	0	0,0333	0,25
176	0	0,025	0	0,0667	0	0
178	0	0	0	0	0	0,125
180	0	0	0	0	0	0,2292
182	0	0	0,675	0,1333	0,2	0
184	0	0	0	0,0333	0	0,1458
186	0,025	0,075	0	0	0	0,0417
188	0,025	0	0	0,1667	0	0,0417
190	0	0,025	0	0	0	0,1042
192	0,275	0	0	0	0	0
196	0,075	0	0,075	0,1333	0,0333	0
200	0	0,175	0	0,1333	0,0333	0
204	0	0,025	0,025	0	0,3333	0
206	0	0,075	0	0,0333	0	0
208	0	0,35	0	0,1333	0,0667	0
210	0,05	0	0	0	0,0333	0
212	0,1	0	0,075	0	0	0
214	0	0	0,15	0	0,1333	0
218	0	0	0	0	0	0,0208
220	0,125	0	0	0	0,1333	0
224	0,125	0,05	0	0	0	0
228	0,075	0	0	0	0	0
232	0,1	0	0	0	0	0
234	0	0,175	0	0,1667	0	0
999	0	0	0	0	0	0,0417

H exp.	0,8575	0,8	0,51	0,8667	0,8044	0,8316
H n.b.	0,8795	0,8205	0,5231	0,8966	0,8322	0,8493
H obs.	0,85	0,7	0,6	10.000	0,9333	0,75

STR-2	AdV	BrV	PiV	BrBG	PiB	SF
(N)	20	20	20	15	15	24
322	0,15	0	0	0	0,0667	0
324	0,025	0	0	0,0333	0	0
326	0,025	0,125	0	0,1333	0,0333	0,0833
330	0,05	0	0	0	0	0,1042
334	0,025	0	0,05	0,0667	0	0
336	0	0	0	0,0667	0	0
340	0	0,025	0	0	0	0
342	0	0	0,125	0,0333	0	0
344	0	0,225	0,075	0	0	0
346	0,05	0,075	0,05	0,1333	0,1667	0
348	0	0	0	0,0667	0,0333	0
350	0,025	0	0,025	0	0	0,0833
352	0,1	0,15	0	0,1667	0,0333	0
354	0	0	0	0,0333	0	0
356	0,075	0	0,05	0	0	0,0833
360	0,325	0,05	0,525	0,1333	0,2	0
362	0	0	0	0,0667	0	0
364	0	0	0	0	0,1	0
366	0	0	0	0	0,1	0
368	0	0	0	0,0667	0,0333	0
370	0	0	0	0	0,0333	0
372	0	0,2	0	0	0,2	0
382	0	0	0	0	0	0,0208
390	0	0	0	0	0	0,1042
396	0	0	0	0	0	0,0417
398	0	0	0	0	0	0,1042
402	0	0	0	0	0	0,2083
416	0	0	0	0	0	0,0208
418	0	0	0	0	0	0,0625
999	0,15	0,15	0,1	0	0	0,0833
H exp.	0,8263	0,84	0,685	0,8933	0,8622	0,8898
H n.b.	0,8474	0,8615	0,7026	0,9241	0,892	0,9087
H obs.	0,65	0,6	0,45	0,6667	0,6	0,6667

Tap2B	AdV	BrV	PiV	BrBG	PiB	SF
(N)	20	20	20	15	15	24
271	0	0,05	0	0	0	0
295	0	0	0	0	0,1667	0
305	0,025	0	0	0	0	0
313	0,4	0,1	0,8	0,3667	0,2	0,125
316	0	0	0	0,0667	0	0
321	0,525	0,85	0,2	0,5667	0,6333	0,7917
999	0,05	0	0	0	0	0,0833

H exp.	0,5613	0,265	0,32	0,54	0,5311	0,3507
H n.b.	0,5756	0,2718	0,3282	0,5586	0,5494	0,3582
H obs.	0,25	0,2	0,3	0,4667	0,7333	0,25

Ssa197	AdV	BrV	PiV	BrBG	PiB	SF
(N)	20	20	20	15	15	24
131	0	0	0,025	0	0	0,0208
133	0	0	0	0,0333	0	0
137	0	0	0,2	0	0	0
143	0	0	0	0,1333	0,2667	0,7917
147	0	0,175	0	0,0333	0	0
157	0,05	0	0,1	0	0,0333	0,1458
161	0	0,025	0	0	0	0
165	0	0,275	0	0,2	0	0
177	0,125	0,225	0,275	0,1	0,2	0
179	0,05	0,125	0,05	0	0,0667	0
181	0	0,125	0,075	0,0667	0,2	0
183	0	0	0,025	0	0	0
185	0,175	0	0,25	0,1333	0,1333	0
189	0,05	0,025	0	0,1333	0	0
193	0,2	0	0	0	0,0333	0
197	0,175	0	0	0,0667	0	0
201	0	0	0	0	0,0667	0
205	0,05	0	0	0,1	0	0
213	0,025	0,025	0	0	0	0
215	0,025	0	0	0	0	0
225	0,025	0	0	0	0	0
999	0,05	0	0	0	0	0,0417
H exp.	0,8688	0,81	0,8025	0,8756	0,82	0,3498
H n.b.	0,891	0,8308	0,8231	0,9057	0,8483	0,3573
H obs.	0,9	10.000	0,9	10.000	0,8667	0,25

Mst60	AdV	BrV	PiV	BrBG	PiB	SF
(N)	20	20	20	15	15	24
94	0,9	0,775	10.000	0,8	0,9333	0,8125
98	0	0,225	0	0,2	0,0667	0,0625
999	0,1	0	0	0	0	0,125
H exp.	0,18	0,3487	0	0,32	0,1244	0,3203
H n.b.	0,1846	0,3577	0	0,331	0,1287	0,3271
H obs.	0	0,35	0	0,4	0,1333	0,125

SfoC79	AdV	BrV	PiV	BrBG	PiB	SF
(N)	20	20	20	15	15	24
103	0,025	0	0	0	0	0
105	0	0	0,025	0	0,0333	0
123	0,875	0,975	0,95	10.000	0,9667	0,875
133	0	0,025	0,025	0	0	0
999	0,1	0	0	0	0	0,125

H exp.	0,2237	0,0487	0,0962	0	0,0644	0,2188
H n.b.	0,2295	0,05	0,0987	0	0,0667	0,2234
H obs.	0,05	0,05	0,1	0	0,0667	0

Sfo8	AdV	BrV	PiV	BrBG	PiB	SF
(N)	20	20	20	15	15	24
192	0	0	0	0	0,0333	0
194	0,075	0	0	0	0	0,4792
196	0	0	0,4	0	0,0333	0
200	0	0,5	0,3	0,2333	0,1667	0,0417
202	0,35	0,45	0,125	0,7667	0,3	0
204	0,4	0	0,05	0	0,3	0
208	0	0	0	0	0,1667	0,3542
226	0,025	0	0	0	0	0
254	0	0	0,025	0	0	0
999	0,15	0,05	0,1	0	0	0,125
H exp.	0,6887	0,545	0,7212	0,3578	0,7622	0,6276
H n.b.	0,7064	0,559	0,7397	0,3701	0,7885	0,641
H obs.	0,35	0,6	0,6	0,4667	0,8	0,3333

Str73INRA	AdV	BrV	PiV	BrBG	PiB	SF
(N)	20	20	20	15	15	24
138	0	0,4	0,075	0	0,0333	0,3958
140	0	0	0	0	0	0,1875
144	0,05	0,025	0	0,2667	0,0333	0,1042
146	0	0,25	0	0	0,1	0,1875
148	0	0	0	0	0,1	0
150	0,2	0,125	0,275	0,4333	0,4667	0
154	0,1	0	0	0	0	0
156	0,05	0	0	0	0	0
160	0,15	0,075	0,625	0,1333	0,1	0
162	0	0	0,025	0	0	0
164	0,3	0,125	0	0,1667	0,1667	0
999	0,15	0	0	0	0	0,125
H exp.	0,81	0,74	0,5275	0,6956	0,7222	0,7465
H n.b.	0,8308	0,759	0,541	0,7195	0,7471	0,7624
H obs.	0,5	0,85	0,5	0,9333	0,8667	0,625

BHMS360	AdV	BrV	PiV	BrBG	PiB	SF
(N)	20	20	20	15	15	24
163	0,125	0	0,05	0	0	0
174	0	0	0,275	0	0	0
177	0	0	0	0,0333	0,1667	0
207	0	0	0,15	0	0,0333	0
209	0,125	0	0	0	0	0
211	0	0,5	0	0,2333	0,0333	0
215	0,05	0	0	0	0	0
217	0	0	0	0	0,0333	0
219	0,075	0,325	0	0,1	0	0

221	0,25	0	0	0	0	0
223	0	0,1	0	0,1667	0	0
227	0	0,025	0	0	0	0
231	0,125	0	0,025	0,0667	0,2	0,4792
235	0	0	0	0	0,1333	0
237	0	0,025	0	0	0,0333	0,2917
239	0	0,025	0,075	0,2333	0,1667	0,0417
241	0,025	0	0,05	0	0	0,1042
243	0	0	0,175	0	0	0
249	0	0	0	0	0	0,0417
259	0	0	0,175	0,0333	0,1	0
305	0,125	0	0	0,0667	0	0
311	0,1	0	0	0	0	0
319	0	0	0	0	0,0333	0
321	0	0	0,025	0	0	0
999	0	0	0	0,0667	0,0667	0,0417
H exp.	0,8562	0,6325	0,8287	0,8378	0,8667	0,6693
H n.b.	0,8782	0,6487	0,85	0,8667	0,8966	0,6835
H obs.	0,9	0,6	10.000	0,8667	0,7333	0,7083

BHMS389	AdV	BrV	PiV	BrBG	PiB	SF
(N)	20	20	20	15	15	24
171	0	0	0	0,0333	0	0
179	0,125	0,3	0,05	0,1333	0,2333	0,1042
181	0	0	0	0	0,0333	0,7292
187	0	0	0	0,1333	0	0
191	0	0,025	0	0,0333	0,1667	0
199	0	0	0,1	0	0	0,1042
205	0	0	0	0	0,0333	0,0208
249	0	0,275	0	0	0	0
255	0,025	0	0,05	0	0	0
257	0,025	0	0,3	0,0333	0	0
259	0,3	0	0	0,0333	0,0333	0
261	0,025	0	0	0,1	0	0
275	0,025	0	0	0,0333	0	0
277	0,075	0	0	0,0333	0	0
281	0,175	0	0,1	0,0333	0	0
283	0,025	0,375	0	0,1333	0,0333	0
285	0,05	0,025	0,125	0,2667	0	0
287	0,075	0	0,275	0	0,1667	0
289	0	0	0	0	0,0667	0
291	0	0	0	0	0,1	0
293	0	0	0	0	0,0667	0
305	0,025	0	0	0	0	0
999	0,05	0	0	0	0,0667	0,0417
H exp.	0,8438	0,6925	0,7937	0,8578	0,8622	0,4444
H n.b.	0,8654	0,7103	0,8141	0,8874	0,892	0,4539
H obs.	0,7	0,7	10.000	0,6667	0,6667	0,3333

CL15589	AdV	BrV	PiV	BrBG	PiB	SF

(N)	20	20	20	15	15	24
196	0	0	0	0	0,0333	0
207	0	0	0,275	0,1	0,0333	0
209	0,15	0,125	0	0	0	0
211	0	0,35	0	0,2	0,0333	0
215	0,05	0	0	0	0	0
217	0,025	0,1	0	0,0333	0,0333	0
219	0,35	0,225	0	0,0667	0	0
223	0,075	0,05	0	0	0	0
225	0	0,05	0	0,1667	0	0
227	0	0,025	0	0	0	0
231	0,125	0	0	0,1	0,3333	0,4167
234	0	0	0	0	0,0333	0
236	0	0	0	0	0,1	0,1458
237	0	0,05	0,05	0,0333	0,0667	0,125
239	0,025	0	0,075	0,1333	0,0333	0,0833
241	0	0	0,3	0,0333	0	0,0625
249	0	0	0	0	0	0,0417
254	0	0	0	0	0	0,0208
257	0	0	0,2	0	0,0333	0
260	0	0	0	0	0,0333	0
301	0,025	0	0	0	0	0
313	0,075	0,025	0,05	0,1333	0,1	0,0625
999	0,1	0	0,05	0	0,1333	0,0417
H exp.	0,8138	0,7925	0,7812	0,8689	0,8378	0,7708
H n.b.	0,8346	0,8128	0,8013	0,8989	0,8667	0,7872
H obs.	0,6	0,65	0,4	0,8	0,7333	0,8333

OMM1121i	AdV	BrV	PiV	BrBG	PiB	SF
(N)	20	20	20	15	15	24
203	0	0,15	0	0	0	0
210	0	0	0	0	0,0333	0
222	0,025	0	0	0,0333	0	0
223	0,125	0	0	0,1	0,0333	0,4792
225	0,025	0	0,075	0	0,3	0,0625
228	0	0,025	0	0	0	0
230	0,15	0	0,1	0	0	0
236	0,4	0,5	0,35	0,4667	0,2667	0
241	0	0	0,325	0	0,2	0
243	0	0,025	0	0,0333	0	0,0208
247	0	0	0	0	0	0,0208
253	0	0	0	0	0	0,0625
255	0	0	0	0	0	0,0208
259	0	0	0,05	0	0	0,0625
261	0	0,025	0,025	0,1	0,0667	0,0417
277	0	0	0	0	0,0333	0,1042
281	0	0	0	0	0	0,0833
312	0,05	0	0	0	0	0
318	0,025	0	0	0,1	0	0
327	0	0,05	0,075	0,0333	0	0
330	0	0,125	0	0,0667	0	0
334	0,15	0,075	0	0	0	0

342	0	0,025	0	0	0	0
999	0,05	0	0	0,0667	0,0667	0,0417
H exp.	0,7725	0,7012	0,7475	0,74	0,7867	0,7361
H n.b.	0,7923	0,7192	0,7667	0,7655	0,8138	0,7518
H obs.	0,9	10.000	10.000	0,9333	0,9333	0,9583

AdV= Adige Valdastico

BrV= Brenta Valdastico

PiV= Piave Valdastico

BrBG= Brenta Bassano del Grappa

PiB= Piave Belluno

SF= Santa Fiora

(N)= number of individuals

In the first column the locus and the allele observed.

H exp.= heterozygosity calculed with bias

H n.b.= heterozygosity calculed without bias (Nei 1978)

H obs. = heterozygosity observed

Appendix E

Complete table for milt concentration and sperm motility data.

Date	Fish ID	Motility	Contamination	Concentration (10^9/ml)	Milt volume (ml)	Volume AquaBoost Dilutor (ml)	Total volume (ml)	Comments
23/11/2015	8053	0	urine and faeces	0,01	5	**-5**	**0**	Piave; empty
23/11/2015	4735	2	none	2,60	5	**2**	**7**	Piave
23/11/2015	8431	1	none	2,35	7	**1**	**8**	Piave; few sperm at the binocular microscope
23/11/2015	1717	3	blood	6,04	5	**10**	**15**	Piave
23/11/2015	2028	0	urine and faeces	0,05	5	**-5**	**0**	Piave; empty
23/11/2015	6077	3	urine and faeces	6,91	3	**7**	**10**	Piave
23/11/2015	8099	3	none	17,50	9	**70**	**79**	Piave
24/11/2015	7234	3	urine	17,05	2	**15**	**17**	Brenta-Valsugana
24/11/2015	5926	0	urine and faeces	2,97	5	**2**	**7**	Brenta-Valsugana
24/11/2015	7202	3	urine	15,48	2	**13**	**15**	Brenta-Valsugana
24/11/2015	6275	2	few urine and faeces	20,00	5	**45**	**50**	Brenta-Valsugana
24/11/2015	9111	3	urine e faeces	17,97	3	**24**	**27**	Brenta-Valsugana
24/11/2015	7468	0	urine and faeces	0,00	1	**-1**	**0**	Brenta (medium variability)
24/11/2015	738	3	none	14,53	2	**13**	**15**	Brenta (medium variability)
24/11/2015	5900	3	none	11,10	10	**46**	**56**	Adige (max variability)
24/11/2015	5379	0	none	6,16	9	**19**	**28**	Adige
24/11/2015	6504	3	very few blood	15,43	8	**54**	**62**	Adige
24/11/2015	6315	2	none	15,05	9	**59**	**68**	Adige
25/11/2015	no chip	3	none	15,03		**0**	**0**	2008-2009 Brenta
25/11/2015	no chip	3	none	18,87		**0**	**0**	2008-2009 Brenta

25/11/2015	632	3	none	19,12		**0**	**0**	2010 Brenta
25/11/2015	202	1	none	8,64		**0**	**0**	2010 Brenta
25/11/2015	699	3	none	14,89		**0**	**0**	2010 Brenta
25/11/2015	363	3	none	5,94		**0**	**0**	2010 Brenta
25/11/2015	741	2	none	13,22		**0**	**0**	2010 Brenta
07/12/2015	7234	3	none	23,67	4	**43**	**47**	Brenta-Valsugana
07/12/2015	7202	3	none	10,86	2	**9**	**11**	Brenta-Valsugana
07/12/2015	9111	3	none	21,03	5	**48**	**53**	Brenta-Valsugana
07/12/2015	8099	3	none	19,93	9	**81**	**90**	Piave
07/12/2015	8431	3	none	16,00	4	**28**	**32**	Piave
07/12/2015	4735	3	none	17,22	6	**46**	**52**	Piave
08/12/2015	801	3	none	15,01	2	**13**	**15**	Piave
08/12/2015	725	3	none	20,95	4	**38**	**42**	Piave
08/12/2015	868	2	none	12,59	2	**11**	**13**	Piave
08/12/2015	356	3	none	16,53	4	**29**	**33**	Piave
08/12/2015	8431	2	none	14,66	1,5	**9**	**11**	Piave
08/12/2015	785	3	none	15,98	1,5	**10**	**12**	Piave
08/12/2015	8053	3	none	13,90	2,5	**15**	**17**	Piave
08/12/2015	610	2	none	17,21	2	**15**	**17**	Piave
08/12/2015	730	3	none	12,69	1,5	**8**	**10**	Piave
08/12/2015	146	3	none	19,96	2	**18**	**20**	Brenta-Valsugana
08/12/2015	363	2	none	17,11	2,5	**19**	**21**	Brenta-Valsugana
08/12/2015	741	3	none	16,26	3	**21**	**24**	Brenta-Valsugana
08/12/2015	no chip	3	few blood	16,29	1,5	**11**	**12**	Brenta
15/12/2015	5900	3	none	20,79	1	**9**	**10**	Adige
15/12/2015	5379	3	none	20,41	1	**9**	**10**	Adige
15/12/2015	6504	3	none	16,35	1	**7**	**8**	Adige
15/12/2015	8099	3	none	17,64		**0**	**0**	Piave (hybrid)

15/12/2015	8431	3	urine	15,48		**0**	**0**	Piave
15/12/2015	4735	2	none	12,00		**0**	**0**	Piave (hybrid)
15/12/2015	7234	2	few urine	21,17		**0**	**0**	Brenta-Valsugana (hybrid)
15/12/2015	9111	3	none	16,70		**0**	**0**	Brenta-Valsugana (hybrid)
15/12/2015	7202	3	few faeces	11,14		**0**	**0**	Brenta-Valsugana
23/12/2015	no chip	3	none	13,99		**0**	**0**	Piave no chip
23/12/2015	no chip	2	none	16,49	0,5	**4**	**4**	Brenta no chip
23/12/2015	no chip	3	none	14,52		**0**	**0**	Brenta no chip
23/12/2015	no chip	3	none	16,14		**0**	**0**	Brenta no chip
23/12/2015	146	3	none	19,65	1	**9**	**10**	Brenta-Valsugana
23/12/2015	356	3	none	16,39	2,5	**18**	**20**	Piave
23/12/2015	725	3	none	14,26	1	**6**	**7**	Piave
23/12/2015	363	3	none	16,28	1	**7**	**8**	Brenta-Valsugana
23/12/2015	202	3	none	19,21	1	**9**	**10**	Brenta-Valsugana
23/12/2015	431	1	none	6,67	3	**7**	**10**	Piave
23/12/2015	no chip	3	none	14,24	1	**6**	**7**	
23/12/2015	610	3	none	21,89		**0**	**0**	Piave
23/12/2015	735	3	none	15,23		**0**	**0**	Piave (hybrid)
23/12/2015	111	3	none	19,31		**0**	**0**	Brenta-Valsugana (hybrid)
23/12/2015	234	3	none	19,64		**0**	**0**	Brenta-Valsugana (hybrid)
23/12/2015	8099	3	urine	19,56		**0**	**0**	Piave (hybrid)
04/01/2016	8099	3	none	18,78		**0**	**0**	Piave (hybrid)
04/01/2016	4735	3	none	7,17		**0**	**0**	Piave (hybrid)
04/01/2016	8431	3	urine	15,09	5	**33**	**38**	Piave
04/01/2016	7234	3	none	13,38		**0**	**0**	Brenta-Valsugana (hybrid)
04/01/2016	9111	3	none	19,83		**0**	**0**	Brenta-Valsugana (hybrid)
04/01/2016	7468	2	none	21,35	6,5	**63**	**69**	Brenta (medium variability)
04/01/2016	7202	2	none	10,62	5	**22**	**27**	Brenta-Valsugana

04/01/2016	6504	2	none	20,23	4,5	**41**	**46**	Adige
04/01/2016	5379	1	none	17,97	2,5	**20**	**22**	Adige
04/01/2016	5900	2	none	20,26	5	**46**	**51**	Adige
04/01/2016	725	2	none	15,40	4,5	**30**	**35**	Piave
04/01/2016	356	3	none	16,22	6	**43**	**49**	Piave
04/01/2016	363	2	none	20,63	4,5	**42**	**46**	Brenta-Valsugana
04/01/2016	785	3	none	14,41	5	**31**	**36**	Piave
04/01/2016	8053	3	none	13,04	7	**39**	**46**	Piave
04/01/2016	6275	3	urine	22,40	5,5	**56**	**62**	Brenta-Valsugana
04/01/2016	1717	2	blood	4,90		**0**	**0**	Piave
04/01/2016	483	1	none	12,19		**0**	**0**	Brenta
20/01/2016	725	2	none	10,32		**0**	**0**	Brenta
20/01/2016	no chip	2	none	17,90		**0**	**0**	adige
20/01/2016	363	3	none	16,70		**0**	**0**	brenta
20/01/2016	nc	2	none	20,31		**0**	**0**	adige
20/01/2016	nc	3	none	20,72		**0**	**0**	adige
20/01/2016	nc	3	none	19,63		**0**	**0**	brenta
20/01/2016	nc	3	none	17,49		**0**	**0**	brenta
20/01/2016	nc	2	none	17,37		**0**	**0**	brenta
20/01/2016	5379	3	none	20,93		**0**	**0**	Adige
20/01/2016	5900	0	none	5,48		**0**	**0**	Adige
20/01/2016	6504	3	none	14,44		**0**	**0**	Adige
20/01/2016	8099	3	none	23,42		**0**	**0**	Piave
20/01/2016	4735	3	none	19,18		**0**	**0**	Piave
20/01/2016	8431	3	none	16,23		**0**	**0**	Piave
20/01/2016	7234	2	none	21,49		**0**	**0**	Brenta-Valsugana
20/01/2016	7202	0	faeces			**0**	**0**	Brenta-Valsugana
20/01/2016	9111	0	empty			**0**	**0**	Brenta-Valsugana

03/02/2016	5379	3	none	15,65	0,5	**3**	**4**	Adige
03/02/2016	5900	2	none	5,20	0,5	**1**	**1**	Adige
03/02/2016	6504	3	none	8,23	0,5	**2**	**2**	Adige
03/02/2016	7468	3	none	8,06	0,5	**2**	**2**	Brenta
03/02/2016	8099	3	faeces	19,49		**0**	**0**	Piave
03/02/2016	7202	1	faeces	7,70		**0**	**0**	Brenta-Valsugana
03/02/2016	4735	3	none	12,59		**0**	**0**	Piave (hybrid)
03/02/2016	7234	3	none	16,30		**0**	**0**	Brenta-Valsugana
03/02/2016	8431	1	urine	6,44		**0**	**0**	Piave

Appendix F

Tables displaying the results of the motility and the milt concentration for the nine individuals monitored during all the reproductive season.

BRENTA

Date	Fish ID	Motility	Concentration (10^9/ml)	Genotype	NOTE
24/11/2015	7234	3	17,05	H	
07/12/2015	7234	3	23,67	H	
15/12/2015	7234	2	21,17	H	
23/12/2015	7234	3	19,64	H	
04/01/2016	7234	3	13,38	H	
20/01/2016	7234	2	21,49	H	
03/02/2016	7234	3	16,30	H	
24/11/2015	7202	3	15,48	P	
07/12/2015	7202	3	10,86	P	
15/12/2015	7202	3	11,14	P	
23/12/2015	7202	3	19,21	P	
04/01/2016	7202	2	10,62	P	
20/01/2016	7202		0,00	P	high contamination
03/02/2016	7202	1	7,70	P	faeces
24/11/2015	9111	3	17,97	H	
07/12/2015	9111	3	21,03	H	
15/12/2015	9111	3	16,70	H	
23/12/2015	9111	3	19,31	H	
04/01/2016	9111	3	19,83	H	
20/01/2016	9111		0,00	H	no milt
03/02/2016	9111		0,00	H	no milt

PIAVE

Date	Fish ID	Motility	Concentration (10^9/ml)	Genotype	NOTE
23/11/2015	4735	2	2,60	H	
07/12/2015	4735	3	17,22	H	
15/12/2015	4735	2	12,00	H	
23/12/2015	4735	3	15,23	H	
04/01/2016	4735	3	7,17	H	
20/01/2016	4735	3	19,18	H	
03/02/2016	4735	3	12,59	H	
23/11/2015	8099	3	17,50	H	
07/12/2015	8099	3	19,93	H	
15/12/2015	8099	3	17,64	H	
23/12/2015	8099	3	19,56	H	

04/01/2016	8099	3	18,78	H	
20/01/2016	8099	3	23,42	H	
03/02/2016	8099	3	19,49	H	
23/11/2015	8431	1	2,35	P	
07/12/2015	8431	3	16,00	P	
15/12/2015	8431	3	15,48	P	
23/12/2015	8431	1	6,67	P	urine
04/01/2016	8431	3	15,09	P	
20/01/2016	8431	3	16,23	P	
03/02/2016	8431	1	6,44	P	urine

ADIGE

Date	**Fish ID**	**Motility**	**Concentration (10^9/ml)**	**Genotype**	**NOTE**
24/11/2015	5900	3	11,10	P	
15/12/2015	5900	3	20,79	P	
04/01/2016	5900	2	20,26	P	
20/01/2016	5900	0	5,48	P	
03/02/2016	5900	2	5,20	P	
24/11/2015	5379	0	6,16	P	
15/12/2015	5379	3	20,41	P	
04/01/2016	5379	1	17,97	P	
20/01/2016	5379	3	20,93	P	
03/02/2016	5379	3	15,65	P	
24/11/2015	6504	3	15,43	P	
15/12/2015	6504	3	16,35	P	
04/01/2016	6504	2	20,23	P	
20/01/2016	6504	3	14,44	P	
03/02/2016	6504	3	8,23	P	

Appendix G

Total results of the egg fertilization experiment.

DATE	FISH ID	RIVER	MOTILITY	CONC 10^9/ml	MILT USED	BORN	DEAD
25-11-15	275	Brenta	2	20,00	Dil	19	281
25-11-15	275	Brenta	2	20,00	Non-dil	130	170
25-11-15	738	Brenta	3	14,53	Dil	53	247
25-11-15	738	Brenta	3	14,53	Non-dil	152	148
09-12-15	801	Piave	3	15,01	Dil	142	158
09-12-15	801	Piave	3	15,01	Non-dil	155	145
09-12-15	725	Piave	3	20,95	Dil	157	143
09-12-15	725	Piave	3	20,95	Non-dil	62	238
09-12-15	431	Piave	2	14,66	Dil	173	127
09-12-15	431	Piave	2	14,66	Non-dil	180	120
09-12-15	363	Brenta	2	17,11	Dil	467	33
09-12-15	363	Brenta	2	17,11	Non-dil	471	29
09-12-15	no chip	Brenta	3	16,29	Dil	286	14
09-12-15	no chip	Brenta	3	16,29	Non-dil	271	29
15-12-15	5900	Adige	3	20,79	Dil	225	75
15-12-15	5900	Adige	3	20,79	Non-dil	224	76
15-12-15	5379	Adige	3	20,41	Dil	214	86
15-12-15	5379	Adige	3	20,41	Non-dil	215	85
15-12-15	6504	Adige	3	16,35	Dil	154	146
15-12-15	6504	Adige	3	16,35	Non-dil	190	110
23-12-15	146	Brenta	3	19,65	Dil	321	79
23-12-15	146	Brenta	3	19,65	Non-dil	296	104
23-12-15	363	Brenta	3	16,28	Dil	219	81
23-12-15	363	Brenta	3	16,28	Non-dil	249	51
23-12-15	725	Piave	3	14,26	Dil	0	300
23-12-15	725	Piave	3	14,26	Non-dil	170	130
04-01-16	468	Brenta	2	21,35	Dil	0	300
04-01-16	468	Brenta	2	21,35	Non-dil	0	300
04-01-16	6504	Adige	2	20,23	Dil	230	70
04-01-16	6504	Adige	2	20,23	Non-dil	275	25
04-01-16	5900	Adige	2	20,26	Dil	15	285
04-01-16	5900	Adige	2	20,26	Non-dil	124	176
04-01-16	356	Piave	3	16,22	Dil	169	131
04-01-16	356	Piave	3	16,22	Non-dil	235	65
03-02-15	6504	Adige	3	8,23	Dil	51	249
03-02-15	6504	Adige	3	8,23	Non-dil	161	139
03-02-15	5379	Adige	3	15,65	Dil	38	262
03-02-15	5379	Adige	3	15,65	Non-dil	238	62
03-02-15	468	Brenta	3	8,06	Dil	45	255
03-02-15	468	Brenta	3	8,06	Non-dil	60	240

Abbreviations:

Dil: Diluted

Non-dil: Non-diluted