University of Algarve

Faculty of Science and Technology

Master´s thesis in Aquaculture and Fisheries

Cytogenetic and histological studies of the Brook trout (*Salvelinus fontinalis*) x Arctic charr (*Salvelinus alpinus*) hybrids

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1. Resumo

A aquacultura tem sido uma actividade em crescendo nos últimos anos, uma vez que o stock de peixe está sobre-explorado e a inovação tecnológica levou ao aumento da produção de peixe.

A aquacultura já tem à algum tempo, um papel importante relativamente às necessidades económicas e sociais, assim como a redução do impacto ambiental. Actualmente, a aquacultura produz cerca de 37% de peixe, quer de origem marinha quer de água doce.

Na Europa, está a registar-se um crescimento da produção de espécies alienígenas, atingindo já, o dobro das espécies indígenas.

Na Polónia, há aproximadamente 600 mil hectares de água doce em tanques, reservatórios, lagos e rios. Este país possui algum desenvolvimento na aquacultura, ocupando aproximadamente 10% de toda a produção em aquacultura na Europa. As espécies mais produzidas são a carpa comum (*Cyprinus carpio*) e a truta arco-íris (*Oncorhynchus mykiss*).

Os salmões (*Salvelinus*) pertencentes à família Salmonidae são os mais estudados devido à sua biologia e ecologia. Actualmente, as espécies mais produzidas na Europa são o salvelino árctico (*Salvelinus alpinus*) e a truta das fontes (*Salvelinus fontinalis).*

O salvelino árctico é das espécies mais bem adaptadas podendo viver em águas muito gélidas, daí a sua distribuição em todo o hemisfério Norte. Pode viver até aos 20 anos. O seu número tem diminuído no estado selvagem, sendo crucial a sua produção em aquacultura. Esta espécie tem uma grande variabilidade ecológica, fenotípica e genética. Os biólogos e cientistas têm um grande interesse nesta espécie devido à sua grande variabilidade geográfica e polimorfismo instável com a intenção de entender a sua microevolução. O seu cariótipo varia entre os 78 e os 84 cromossomas dependendo da sua geografia.

A truta das fontes é uma espécie endémica do continente norte-americano, no entanto tem sido introduzida em diversas partes do mundo, inclusive o Norte da Europa. Estes peixes preferem viver em águas mais oxigenadas, de preferência em lagos e ribeiros. A truta das fontes é a espécie de *Salvelinus* que tem o tempo de vida mais curto, no entanto a sua longevidade é variável dependendo se é nativo ou introduzido. Relativamente ao alimento, esta espécie é oportunista, alimentando-se de vários organismos, dependendo da disponibilidade do alimento. Esta espécie é bastante

tolerante às diferentes condições ambientais, como a baixa temperatura, pouco alimento ou baixos valores de pH. Ao contrário do salvelino árctico, o seu cariótipo é estável com um número diplóide 2n= 84.

A hibridação consiste no cruzamento de dois seres vivos, seja plantas ou animais, da mesma espécie ou de espécies diferentes. Os aquacultores utilizam esta técnica em alguns peixes para produzir indivíduos com as características pretendidas ou melhorar a performance dos peixes, como por exemplo a resistência à doença, aumento da taxa de crescimento, qualidade da carne ou produção de peixes estéreis. A produção de peixes estéreis possibilita que não haja trocas genéticas em situações de acidente.

O salvelino árctico e a truta das fontes podem hibridizar em condições naturais, existindo até híbridos em alguns rios no Sul da Europa.

Os salmonídeos são um grupo com uma grande variabilidade cromossómica, quer intra como inter-especies. Fenómenos como re-arranjos cromossómicos ou eliminação dos mesmos podem influenciar essa variabilidade, uma vez que durante a evolução do ancestral comum foram sujeitos a tais fenómenos. Estes mecanismos podem ter influência na viabilidade dos híbridos.

Estudos citológicos e histológicos poderão ajudar a perceber a viabilidade dos híbridos de truta das fontes e salvelino árctico. Neste estudo, foram colhidos alguns exemplares de híbridos destas duas espécies para realizar tais análises citológicas e histológicas.

Com a realização do cariótipo destes exemplares foi possível contar, analisar e comparar os cromossomas entre eles e com os seus progenitores. O número de cromossomas alcançado foi variável, sendo que o tipo de cromossomas também poderá influenciar a sua viabilidade.

Também foram colhidas algumas amostras das gónadas de alguns exemplares de forma a identificar o sexo e a sua viabilidade, tendo como a intersexualidade de alguns híbridos um resultado inesperado.

Uma das grandes questões deste trabalho é tentar perceber as razões de alguns indivíduos apresentarem esterilidade. O macho, híbrido resultante do cruzamento entre a égua e um burro, é um dos exemplos mais conhecidos, sendo este um exemplo de híbrido estéril. Esta esterilidade poderá estar relacionada com o número impar de cromossomas (2n= 63) provenientes dos progenitores, égua com número diplóide de 64 cromossomas e o burro com 62.

Com estas espécies de salmões, o mesmo poderá ter ocorrido, sendo que o retrocruzamento poderá ter influenciado a esterilidade.

Assim sendo, neste trabalho sugerimos as possíveis causas para a variação do número de cromossomas dos híbridos de truta das fontes com os salvelinos árcticos, tal como a viabilidade dos mesmos e o desenvolvimento das gónadas dos híbridos através de análises histológicas.

2. Abstract

In aquaculture, application of fish hybrids has increased. This technique permits improvement of the fish production by providing specimens showing better growth rate when compared to the parental species. Indeed, sterile individuals are highly demanded because quite frequently parental fish mature before they reach the market size, which impairs their growth and decrease their economic value. Throughout the last years, the commercial and scientific interest in salmonids has increased rapidly, among them, the brook trout (*Salvelinus fontinalis*), Arctic charr (*Salvelinus alpinus*) are species that can be crossed to produce hybrids that might by cultured in the fish farms. In the present thesis, we have assessed chromosome numbers and evaluate gonadal sex in the brook trout X Arctic charr hybrid progenies.

In our populations, the karyotype of the brook trout comprises 84 chromosomes: 16 bi-armed chromosomes (meta-submetacentric) and 68 one-armed chromosomes (telo-acrocentrics) and the chromosome arm number, NF= 100. Arctic charr karyotype shows variation related to the chromosome number (2n= 81-82) and stable chromosome arm number (NF= 100). 2n= 81 chromosomes consisted of 19 bi-armed and 62 one-armed chromosomes, while 2n= 82 karyotype was organized into 18 meta-submetacentric and 64 acrocentrics.

The cytogenetic and histological analysis of the brook trout X Arctic charr hybrids (sparctics) was carried out to asses chromosome and chromosome arm number and gonadal sex of the studied specimens. Diploid chromosome number in the hybrids varied from 81 to 84 and individuals with 83 and 84 chromosomes were predominant. Most of the fish had chromosome arm number equal to 100. Robertsonian fusion in the Arctic charr and chromosome behaviour in the hybrid fish cells might lead to the observed variation in chromosome numbers in the hybrids.

Among studied fish, 12 were males, 3 were females and 9 had intersex gonads. No correlation between chromosome number and disturbances in the gonadal development was found. This might suggest that intersex gonads might have been developed as a consequence of disturbances in the genetic sex determination process. Genetic sex determination acts properly in the parental species but in the hybrids this may not be as efficient.

3. Introduction

Aquaculture is defined as the farming of aquatic organisms, including fish, crustaceans, molluscs and aquatic plants, where man has some kind of intervention in the rearing process to enhance production, such as feeding, stocking and protection from predators (Gomiero, Giampietro et al. 1997).

The production of fish by aquaculture has increased rapidly in the last years. This increase parallels an increase in sustainability problems in the global fisheries. The worldwide market growth for marine species, the changing consumer preferences in developed countries, the overexploitation of fish stocks and technological innovations led to an increase in aquaculture production (Frankic and Hershner 2003; FAO 2005; Millar and Tomkins 2007). Aquaculture aims to respond to human needs at the economic and social levels as well as to reduce the environmental impact and protect all the natural resources (Frankic and Hershner 2003).

Presently, aquaculture produces around 37% of the aquatic finfish food of marine and freshwater origin in the world. According to FAO, in 2008, Asia dominated world aquaculture production (Table 1) because of China's contribution of about 60% of world production (FAO 2010).

In the European Union (EU), fishing and aquaculture are some of the most important economic activities; however they remain well below production in other continents. EU contributes only with 4.5% of world production.

Currently, European inland aquaculture is dominated by introduced species from other continents, such as rainbow trout, brook trout or silver carp. For a finfish, the production of introduced species is almost double that of indigenous species (Turchini and Silva 2008). There are several reasons that led to the introduction of new fishes in Europe, as improvement of wild stocks, aquaculture, ornament, sport, biological control or accident (Elvira 2001).

Table 1 – World aquaculture production (tonnes) in 2000 and 2008. Data do not include aquatic plants (Source: FAO 2010).

Continents	2000	2008
Africa	399.788	940.440
America	1.422.647	2.405.166
Asia	28.400.213	46.662.031

Europe	2.072.160	2.366.354
Oceania	121.312	172.214

Poland has about 600.000 ha of freshwater reservoirs, ponds, lakes, rivers and approximately 524 Km of coastline (FAO 2011). Poland is a country with high level of aquaculture development, containing over 1.000 farms and inland fishery sites (CSN-INTRAN 2005) contributing 9.3% of the entire European inland aquaculture finfish production. In the last decade, Polish aquaculture production has increased exceeding the barrier of 30.000 tons (Fig. 2) mainly due to the production of the common carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*) (Turchini and Silva 2008). The increase in trout production was due to new trout culture facilities, modern equipment, new culture methods, balanced trout feed, more care with the health of fish and growing market for this species in Poland. The earth ponds are the most commonly used in polish farms (FAO 2011).

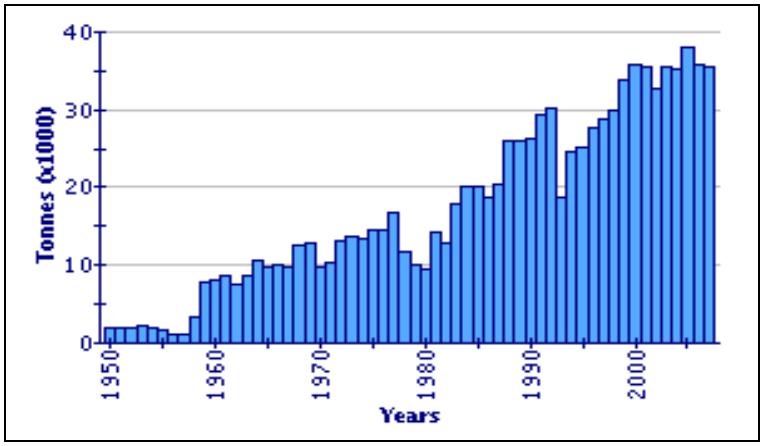

Figure 2 - Poland Aquaculture Production (Source: FAO Fishery Statistic, 2010).

Moreover, salmonid fishes production has been developed for restocking purposes. In the last three decades there has been an intense growth of restocking and initiation of sea-ranching programs (Pennell and Barton 1998). Salmonid fishes other than rainbow trout have great potential and might be produced as farmed animals in Polish aquaculture. The salmonid fishes trade has experienced a spectacular growth in recent years. The interest in this group is due to the recreational and commercial value of some species as rainbow trout, Atlantic salmon and brook trout, and they are becoming increasingly important as models for ecological and evolutionary questions. Many studies have been done in salmonids, such as comparative analyses of salmonid adaptations, comparative genomics, evaluation of conservation priorities and studies

involving inference of ancestral states (Crespi and Fulton 2004; Jankun, Kuzminski et al. 2007).

Apart from the rainbow trout and Atlantic salmon, charrs (*Salvelinus* sp.) are another salmonid species with potential for being "aquacultured". This interest is explained by the charrs biology and ecology. Charrs have a Holarctic distribution including American, Asian and European continents, in different aquatic environments such as marine and freshwater habitats. In the past, charrs suffered repeated isolation and re-contact between divergent lineages influenced by topographic and climate changes associated with Pleistocen glaciations. The existence of a large morphological, ecological and genetic variability within species of the genus *Salvelinus*, has made these fishes excellent models in evolutionary terms as well. Habitat changes and overexploitation, associated with the fact that they occupy reduced aquatic habitats, may lead them to extinction (Magnan, Audet et al. 2002). Currently, charr species farmed in the European continent are Arctic charr *S. alpinus* (Linnaeus, 1758) and brook trout *S. fontinalis* (Mitchill, 1814), but only the first one is originally from Europe (Haffray 2008).

Arctic charr (*S. alpinus)* (Fig. 3) has the most northern distribution (Fig. 4) of the whole salmonids family and may be well adapted to cold waters.

Figure 3 – *S.alpinus* from andromous population, in Norway (Source: Handbook of European Freswater Fishes, 2007).

Arctic charr has high capacity of adaptation to different habitats and they can live in oligotrophic lakes, streams and in the sea (Klemetsen, Amundsen et al. 2003). Aquaculture production has been the solution for the decrease of the wild fish stocks of Arctic charr.

Figure 4 - Arctic charr world distribution (Source: Oceana – Protecting the World´s Oceans, 2010).

Arctic charr have a large ecological and phenotypic variability. Mature fish may vary between 3kg and 12kg, the coloration varies very much in charr, being the most colourful of all the northern straights. Their diet also varies according to seasonal changes. Cannibalism may be present in some cases, even beneficial for developing and maintaining bimodal size distributions (Klemetsen, Amundsen et al. 2003; Kuttner, Moghadam et al. 2011).

This species usually can live up to 20 years, but the maximum observed was 32 years. Most Arctic charrs is anadromous and they grow faster than the lacustrine and riverine stocks. Usually, they spawn in autumn (from October to December). Males are territorial and may mate with several females (Kottelat and Freyhof 2007). The interest of scientists to study the Arctic charr is due to their geographic variability, sympatric forms and subsequent unstable polymorphism, with the aim of understanding the structure of the group and its microevolution (Alekseyev, Samusenok et al. 2002). With respect to karyotype, the Arctic charr is quite variable, ranging from 2n= 78 to 2n= 84, depending on his geography (Gjedrem, Eggum et al. 1977; Phillips and Ihssen 1985; Phillips, Pleyte et al. 1988; Hartley 1989; Pomianowski, Jankun et al. 2012).

Brook trout (*Salvelinus fontinalis*) (fig. 5) is endemic in the North American continent (fig. 6). However, brook trout has been introduced throughout the world, especially in Central and Northern Europe. This species lives in well oxygenated lakes and streams preferring temperatures below 20º (Naiman, McCormick et al. 1987).

Figure 5 – *Salvelinus fontinalis* from German Aquaculture (Source: Handbook of European Freswater Fishes, 2007).

Brook trout is the charr with shortest lifespan (Ficke, Peterson et al. 2009), however the longevity of the species is variable, depending on whether they are native or introduced. Introduced species can reach 15 years of age. Brook trout is a fish that spawns in late summer or early autumn, in the headwater streams and rivers. In southern Europe, it reaches sexual maturity between 1-2 years old and in northern Europe usually between 3-4 years old and they can weigh up to 5kg (Naiman, McCormick et al. 1987; Kottelat and Freyhof 2007). Brook trout is an opportunistic species, feeding on various organisms, depending on prey availability. This salmonids are tolerable to a wide

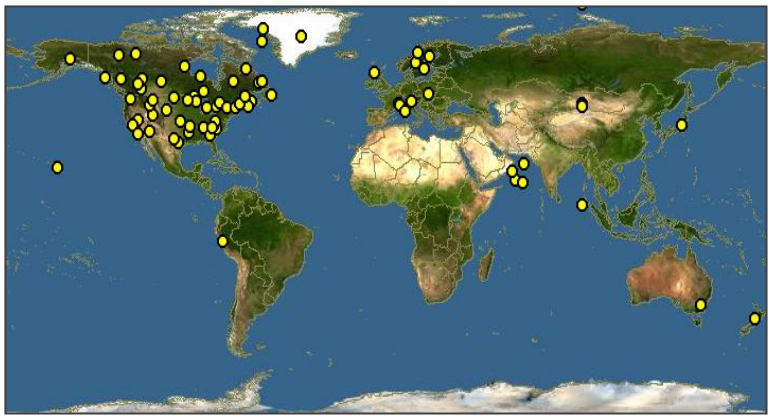

Figure 6 - Brook trout world distribution (Source: www.discoverlife.org, 2010).

variety of environmental conditions such as low temperature, low food and resistant to low pH (Naiman, McCormick et al. 1987). In contrast to Arctic charr, the brook trout karyotype is stable with diploid chromosome number 2n= 84 and chromosome arm number NF= 100 (Woznicki and Kuzminski 2002).

A limiting factor for the development of the brook trout aquaculture is the early maturation. Production of sterile triploid brook trout or hybrid diploid and triploid brook

trout X Arctic charr fish may solve the problem of early maturation (Woznicki and Kuzminski 2002; Basçinar and Okumus 2004).

Hybridization is a strong support for understanding the biological principles of the studied species and their knowledge in conservation and living resource management (Epifanio and Nielsen 2001), and is also a very promising genetic approach utilized in aquaculture to produce fish showing required features (Bartley et al. 2001). Hybridization is a genetic cross that may involve individuals within species or from different species. This breeding technique is used by fish farmers in the hope of producing aquatic organisms with specific desirable traits or general improvement in performance such as disease resistance, increasing growth rate, meat quality and production of sterile animals (Bartley, Rana et al. 2001). Hybridization might be thought as a tool to produce sterile fish in the case of several species such as female rainbow trout (*Oncorhynchus mykiss*) and male masu salmon (*Oncorhynchus masou masou*) (Zheng, Tanaka et al. 2011), brown trout x brook trout (Bartley, Rana et al. 2001) and white bass (*Morone chrysops*) x striped bass (*Morone saxatilis*) (Bartley, Rana et al. 2001).

Arctic charr and brook trout can hybridize in natural conditions (hybrids of the brook trout and Arctic charr are called sparctics) (Gross, Gum et al. 2004) and such hybrids are usually fertile (Johnson, J. E. Wright et al. 1987). However, among matured sparctics obtained in the Department of Salmonid Research, Inland Fisheries Institute in Olsztyn, Rutki, Poland, individuals with decreased fertility or even fish that are unable to produce any gametes have been observed (about 20-30% of the hybrid offspring, personal communication – Dobosz, Department of Salmonid Research). Such fish have been thought to have reduced gonads and able to produce only few if any gametes. Or these individuals were intersex hybrids with disturbed gamete production process. Sterility in fish is observed in the triploid individuals, among others. Galbreath and Thorgaard (1995) observed that female hybrids (Atlantic salmon X brown trout) backrossed with Atlantic salmon were viable, triploid and sterile. In aquaculture, sterility offer is an advantage especially in fish that mature early before reaching expected market size.

We cannot exclude that handicapped gamete production or production of aneuploid gametes in the hybrids are consequences of the disturbances in the meiotic divisions. Especially when parental individuals differ in chromosome numbers. Salmonids show huge interspecies and intraspecies variation in chromosome number

and structure, described as centric, tandem fusions and inversions in *Salmo, Salvelinus, Oncorhynchus, Coregonus* species (May, Johnson et al. 1989; Hartley 1991; Frolov 1995; Philips and Ráb 2001). Listed rearrangements have played an important role in the evolution of salmonid karyotypes during rediploidization following whole genome duplication experienced by the salmonid ancestor (Allendorf and Thorgaard 1984). This has been proposed to be related to the differences between brook trout and Arctic charr karyotypes. Brook trout specimens from the studied stocks show karyotype composed of 84 chromosomes (FN= 100), Arctic charr are characterized by a variable chromosome number that ranged from 2n= 81 to 2n= 82 with fundamental chromosome number, NF= 100 and karyotypes composed of 19 meta-submetacentrics and 62 acrocentrics (NF= 100), and 18 bi-armed chromosomes and 64 acrocentrics (NF= 100), respectively. Such polymorphism has been proposed to be related to the Robertsonian fusion. This translocation involves fusion of two acrocentric chromosomes to form a metacentric chromosome but the chromosome arm number remains constant, very usual in salmonid fishes (Disney and Wright 1987; Hartley 1989; Philips and Ráb 2001).

Gonadal intersex in hybrid specimens may be related to disturbances in the process of sex determination. The development of sexual characteristics in vertebrates is usually determined by the sex determining system and includes sex chromosomes and sex determining gene(s). Usually, Salmonidae males are heterogametic (XY) and females are homogametic (XX) (Phillips, Konkol et al. 2001; Phillips, Matsuoka et al. 2002; Woram, Gharbi et al. 2003). In the brook trout, sex chromosomes are the medium sized metacentrics and the X chromosomes has a terminal heterochromatic band on the short arm which is missing in the Y chromosomes (Phillips et al. 2001; Phillips et al. 2002; Ocalewicz et al. 2004). In the Arctic charr, sex chromosomes are not well morphologically differentiated (Phillips, Matsuoka et al. 2002; Woram, Gharbi et al. 2003). Although sex determination systems work efficiently in the parental species, in their hybrids errors may happen.

On top of that, teleosts have a very labile process of sex differentiation that may be disturbed by many factors including steroid hormone treatments or water pollutants called endocrine disruptors. Pollution has been suggested as a cause of intersexualism in several salmonids as well (Kinnison, Unwin et al. 2000). However, in the case of hybrids studied in the present thesis this last option is the least probable.

To attempt to discover what could be responsible for the decreased fertility in some of the sparctics, we decided to undertake karyological and histological analysis of

the spartic specimens. The first objective of the present study was to provide basic information about sparctics karyotype. The second objective was to check the status of the gonadal development of the hybrids by an histological approach. Comparison of the karyological and histological result was the third objective of this thesis.

4. Materials and methods

4.1. Sampling

Brook trout oocytes and Arctic charr spermatozoa were collected from the broodstocks kept at the Department of Salmonid Research, Inland Fisheries Institute in Olsztyn, Rutki, Poland in November 2008. Oocytes were collected from four randomly chosen brook trout females and spermatozoa derived from one Arctic charr male.

Oocytes were pooled and inseminated with Arctic charr semen. Hybrid progenies hatched in February 2009. Hybrids from Sf x Sa 1 to Sf x Sa 6 were sampled after 8 months of rearing and hybrids from H13 to H34 were sampled after 15 months of rearing for cytogenetic and histological analysis.

4.2. Preparation of Metaphase Chromosomes

Direct "in vivo" chromosome preparation method (so called air drying technique) was applied to provide metaphase chromosomes from twenty eight hybrid individuals. The fishes were injected intraperitoneally with 0.01% of $CoCl_2$ (Cobalt Chloride) solution (0.2mg/100g body weight). After 3 days, fish were injected 0,01% colchicine solution (1mg/100g body weight). After 150 minutes, fish were sacrificed with overdose of MS-222 anesthetic and decapitation. Fish kidneys were sampled, homogenized and placed in 15 ml centrifuge tubes in prewarmed 0.075M KCl (Potassium Chloride) solution. After 45 minutes of hypotonization in this solution, samples were centrifuged at 1000 rpm (10 minutes). Supernatant was poured off and 5 ml of fixative (3:1 methanol/acetic acid) were added, drop by drop, shaking gently the tube at room temperature (10 minutes). Afterward, suspended cells were centrifuged at 800 rpm (5 min) and the supernatant was poured off. The last two steps were repeated twice separated by 30 minutes break. One to three drops of cells suspended in the fixative were dropped on the clean microscopic slides. Chromosomes were initially stained in buffered Giemsa (10%, 10 minutes) for visualization and description of the chromosomal morphology. Moreover, chromosomes were stained with 4, 6-diamidino-2-phenylindole DAPI for identification of AT-rich chromatin regions. Three drops of antifade solution Vectashield (Vector, Burlingame, USA) containing DAPI (1.5 µg /ml) were dropped onto a slide and covered with a coverslip (Ocalewicz et al. 2004; Pomianowski et al. 2012).

4.3. Karyotyping

Metaphase plates were analyzed under two microscopes: a Nikon Optiphot microscope equipped with a fluorescent lamp and Nikon digital camera (1) and a Zeiss Axio Imager A1 microscope equipped with a fluorescent lamp and a digital camera. Images were captured and the electronic processing of the images was performed using Band View/FISH View software (Applied Spectral Imaging) (2).

Pictures were analyzed using computer and program Adobe Photoshop CS4. Chromosome were cut and placed according to their homology, size and centromere position for karyotyping.

In individual H34, it was not possible to observe any metaphase plates due to the poor quality of the chromosomal preparations. Individuals Sf x Sa 1 and H27 showed poor morphological quality of the metaphase spreads and thus it was only possible to evaluate the chromosome number.

4.4. Histology

Histological analysis of the karyologically studied hybrid individuals was carried out to confirm the gonadal sex of the fish. Pieces of gonads were collected and fixed in Bouin's solution. Subsequently, the tissues were dehydrated in alcohol with increasing concentration, fixed in xylene and left intact for paraffin embedding. Slices of 4-5µm thick were cut using a rotational microtome model RM 2155 (LEICA Microsystems, Wetzlar, Germany), stained with the haematoxylin and eosin topographic method and the Mallory method (Humason 1970). Histological analyses of cross-sections for the shape, size and the type of germ cells present in gonads were conducted by light microscope ECOTONE with classical micro image computer analysis software MultiScanBase version 8.0 for WINDOWS (Computer Scanning Systems Ltd.).

In individuals Sf x Sa 1, H25, H28 and H29, gonads were not found.

5. Results

Brook trout individuals from this broodstock are characterized by diploid chromosome number equalled 84 and the karyotype composed of 16 bi-armed chromosomes and 68 one-armed chromosomes. In Arctic charr, the diploid chromosome number in the studied individuals was 2n= 81 and 2n= 82 with fundamental arm number (NF) equaled 100 what was proposed to be related to the Robertsonian fusions (Pomianowski et al. 2012).

5.1. Karyotyping

Metaphase plates from the studied individuals were scored (Table 7). Apart from Sf x Sa 1, H27 and H34 specimen, it was possible to observe high quality metaphase spreads and assess number and morphology of the chromosomes in all individuals. Quality of the chromosomes enabled identification of metacentric and acrocentric chromosomes in most of the studied fish.

Chromosome number in the studied specimens varied from 81 to 84 with the predominance of 83 and 84 chromosomes (Table 7, Fig. 8). Sf x Sa 4 was the one with 81 chromosomes but it had only 3 metaphases to be analyzed. Nine of 27 hybrids studied had less than 10 metaphases observed and only three had more than 30 metaphases.

Most individuals had 16 or 17 metacentric chromosomes (twenty-two out of twenty five) in their karyotypes.

Metaphase plates with different chromosome number or different chromosome arm number were chosen to be presented (Fig. 8). Hybrid H33 contains 82 chromosomes, with 17 metacentric, 64 acrocentric and one subtelocentric chromosome, totaling 99 chromosome arm number (Fig. 8A). H32 has 83 chromosomes divided by 17 metacentric, 65 acrocentric and one subtelocentric chromosome, with NF= 100 (Fig. 8B). Hybrid H23 has 84 chromosomes, with 17 metacentric, 66 acrocentric and one subtelocentric chromosome, totaling 101 chromosome arm number (Fig. 8C). Hybrid Sf x Sa 5 contains 84 chromosomes divided by 16 metacentric and 68 acrocentric chromosomes with NF= 100 (Fig 8D).

Table 7 – Gonadal sex, diploid chromosome number and chromosome arm numbers of the hybrids. The sampling of Sf x Sa 1 to Sf x Sa 6 was made in autumn (8 months after hatching) and H13 to H34 was made in spring (15 months after hatching).

Fish	Sex/histology	Modal chromosome number	Meta/Sub metacentric	Acrocentric/ subtelocentric	NF	Number of plates
Sf x Sa 1	No Histology	2n= 83	-	-	-	4
Sf x Sa 2	Intersex	2n= 84	16	68	100	7
Sf x Sa 3	Male	2n= 83	17	66	100	8
Sf x Sa 4	Male	2n= 81	19	62	100	3
Sf x Sa 5	Male	2n= 84	16	68	100	13
Sf x Sa 6	Male	2n= 84	16	68	100	19
H 13	Intersex	2n= 83	17	66	100	13
H 14	Male	2n= 82	17	65	99	16
H 15	Male	2n= 84	16	68	100	30
H 16	Intersex	2n= 82	17	65	99	12
H 17	Female	2n= 83	17	66	100	11
H 18	Intersex	2n= 83	17	66	100	14
H 19	Female	2n= 82	18	64	100	3
H 20	Intersex	2n= 83	17	66	100	13
H 21	Male	2n= 84	16	68	100	2
H 22	Male	2n= 83	16	67	99	15
H 23	Intersex	2n= 84	17	67	101	42
H 24	Male	2n= 84	16	68	100	52
H 25	No Histology	2n= 82	17	65	99	8
H 26	Male	2n= 83	17	66	100	7
H 27	Intersex	2n= 83	-	-	-	3
H 28	No Histology	2n= 84	16	68	100	15
H 29	No Histology	2n= 84	16	68	100	20
H 30	Male	2n= 84	16	68	100	25
H 31	Intersex	2n= 84	18	66	102	18
H 32	Male	2n= 83	17	66	100	21
H 33	Female	2n= 82	17	65	99	97
H 34	Intersex	-	-	-	-	-

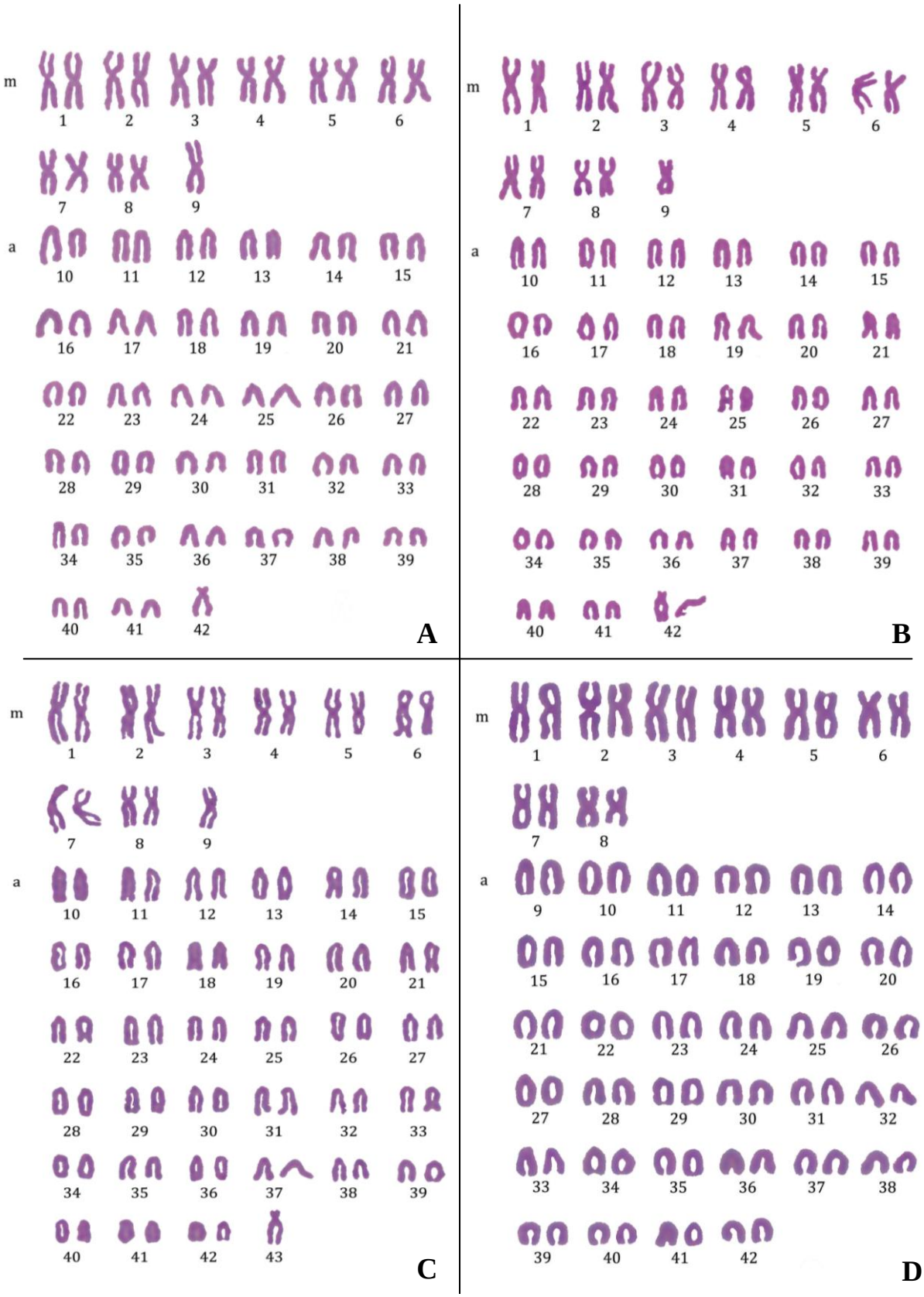

Figure 8 – Karyotypes of the hybrid individuals. **A** – H33 - female (2n= 82), **B** – H32 - Male (2n= 83), **C** – H23 - Intersex (2n=84) and **D** – Sf x Sa 5 - Male (2n=84). **m** – Metacentric and submetacentric, **a** – acrocentric and telocentric

5.2. Histology

In the course of the histological analysis, gonadal sex was detected in most of the hybrid specimens. Individuals H17, H19 and H33 were females. Ovaries were more easily identified even at the earliest sampling stage as they were larger and the developing oogonia visible (Otto 1995) (Fig. 9). The different size of the oocytes was attributed to different stages of their maturation (Fig. 9).

Spermatogonia and spermatids were observed in individuals Sf x Sa 3, Sf x Sa 4, Sf x Sa 5, Sf x Sa 6, H14, H15, H21, H22, H24, H26, H30 and H32 (Fig. 10).

Both oogonia and spermatogonia were present in individuals Sf x Sa 2, H13, H16, H18, H20, H23, H27, H31 and H34 (Fig. 11).

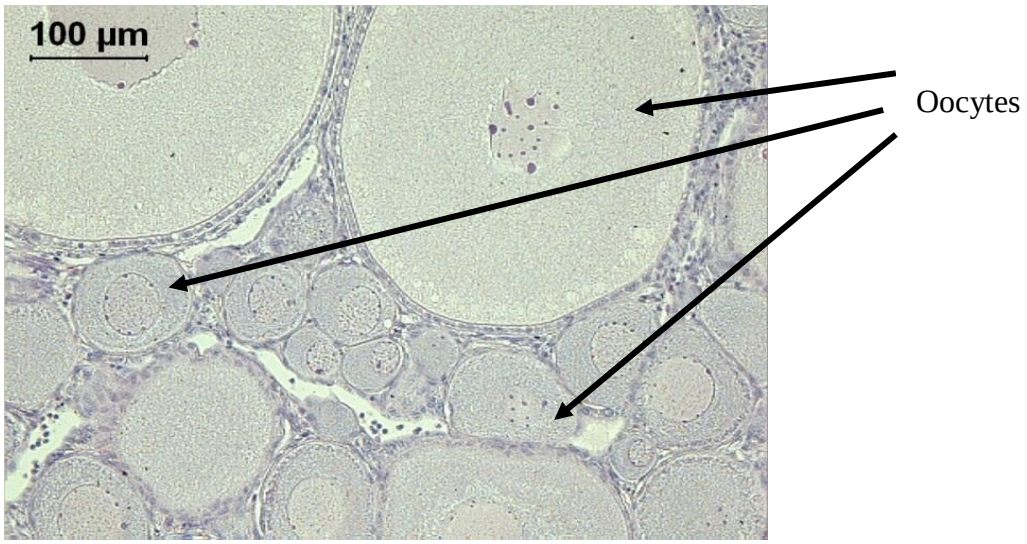

Figure 9 – Histological cross-section of sparctic charr ovaries stained by HE method.

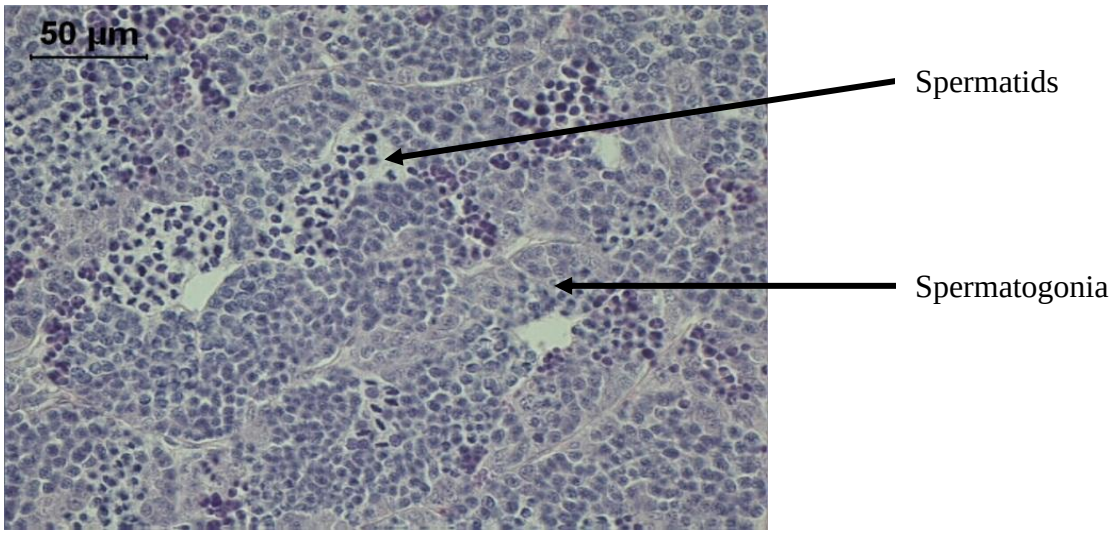

Figure 10 - Histological cross-section of sparctic charr testis stained by HE method.

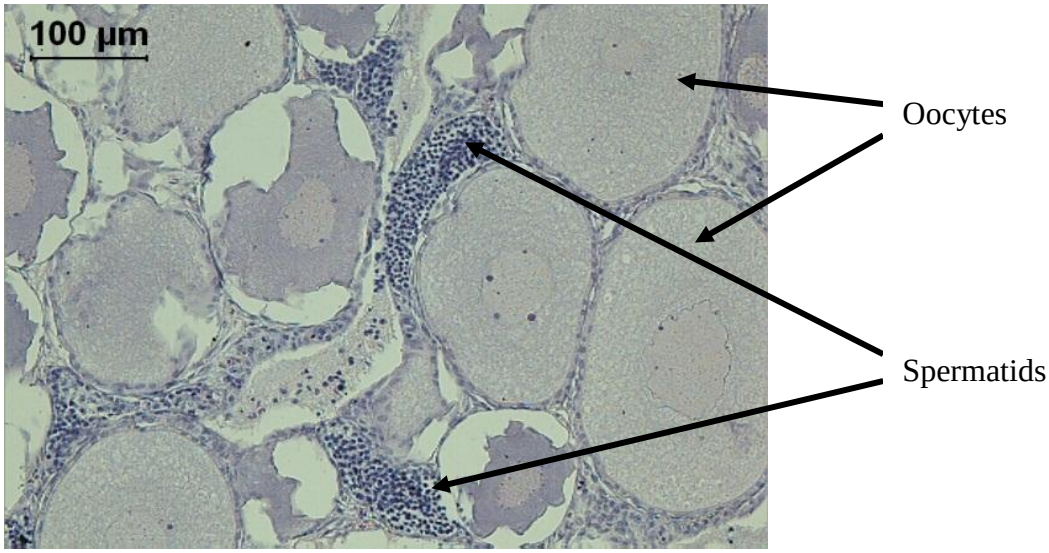

Figure 11 - Histological cross-section of intersex sparctic charr stained by HE method.

6. Discussion

Fish hybridization is a process with several important applications in aquaculture including the increase of individual growth rates, harvestability and environmental tolerance, production of sterile individuals and combination of desirable characteristics from two species in a single individual (Bartley, Rana et al. 2001). In general, hybrids can be unviable, viable and fully fertile and/or viable but showing decreased fertility or even sterile (e.g. mule). Sterility in the male mules (2n= 63) was related to different diploid chromosome number of the parents, horse (*Eqqus caballus*) with 64 chromosomes and donkey (*Equus asinus*) with 62 chromosomes and difference of chromosome complement, the horse had 19 pairs of metacentric autosomes and 18 pairs of acrocentrics, whereas donkey had 19 and 11, respectively (Trujillo, Stenius et al. 1962). In fish Esox hybrids (muskellunge *Esox masquinongy* and pike *Esox lucius*) and bass hybrids (white bass *Morone chrysops* and striped bass *M. saxatilis)* are sterile (Bartley, Rana et al. 2001).

High mortality among hybrid progenies of the particular parental fish individuals may be related to the incompatibility between the maternal cytoplasm in the oocytes and paternal genome, which may lead to partial or total elimination of chromosomes from one of the progenitors and sometimes may end up in the mortality during the embryonic development. This has been observed in the case of Japanese charr (*Salvelinus leucomaenis*) x Chum salmon (*Oncorhynchus keta*) hybrids (Goodier, Ma et al. 1987) and the hybrid progenies of female Masu salmon (*Oncorhynchus masou*) and male rainbow trout (*Oncorhynchus mykiss*) (Fujiwara, Abe et al. 1997).

However, there are some cases where fish hybrids can be both, viable and fertile. This has been observed among salmonid, cyprinid, Tilapia and catfish species (among others) (Bartley et al. 2001). A cross between brown trout (*Salmo trutta*) (2n= 78-84, NF= 98-102) and Atlantic salmon (*Salmo salar*) (2n= 54-58, NF= 72-74) in rivers in southern Europe has been observed quite frequently. Mating strategies and contact between isolated species may lead to the breakdown of reproductive barriers causing introgression and hybridization. This can induce deliberate or accidental releases of individuals in the wild and consequent alteration of native genetic pool (Castillo, Ayllon et al. 2008). In Germany, fish farmers produce fertile hybrids between Arctic charr and brook trout (Gross, Gum et al. 2004). Although Ma and Yamazaki (1986) consider that differences in the number of chromosomes between two parental species has less impact

on the viability of hybrids than genetic distance, the odd number of chromosomes in the hybrid progenies might be the cause of decline in fertility or even sterility in some fish hybrids.

Hybrids resulting from salmonid species of the same genus, such as Masu salmon (*Oncorhynchus masou*) (2n= 66, NF= 100) x Pink salmon (*Oncorhynchus gorbuscha*) (2n= 52-54, NF= 100) (Arai 1984), although expected to be unable to survive due the interspecific karyotype differences show, high degree of viability (Galbreath and Thorgaard (1995).

The number of chromosomes in the brook trout is stable and equal to 84 (Ueda and Ojima 1983; Woznicki and Kuzminski 2002). On the other hand, the Arctic charr show chromosome variations, both within and between populations. In fact, variations in the diploid chromosome number (2n), as well as the number and size polymorphisms of differentially stained heterochromatin have been observed by several authors (Hartley 1989; Pomianowski, Jankun et al. 2012). For instance, the most common diploid chromosome number of Arctic charr from North America is 2n= 78 (Phillips, Pleyte et al. 1988; Hartley 1989; Phillips, Pleyte et al. 1989), while fish from other regions may have 2n= 80 (Gjedrem, Eggum et al. 1977) or 2n= 82 (Phillips and Ihssen 1985). Variation in the number of the Arctic charr chromosomes has been also observed among individuals from the Rutki strain (2n= 81 to 82). Individuals with 81 and 82 chromosomes had the same chromosome arm number (NF= 100) which suggested that differences are due to Roberstonian fusion (Pomianowski et al. 2012).

Salmonid karyotypes evolve through the Robertsonian translocations/tandem translocations and pericentric inversions leading to decrease of the total chromosome number. This is related to rediploidization process following the round of the whole genome duplication experienced by the salmonid ancestor (May, Johnson et al. 1989; Hartley 1991; Frolov 1995; Philips and Ráb 2001), however, there is no consensus about salmonids ancestor karyotype. Whereas some studies indicate that after genome duplication salmonids ancestor had about 112 to 156 chromosomes (Zelinsky and Makhrov 2002), others indicate that they had either 2n= 100 and NF= 100 (Frolov 1995) or 96 chromosomes and NF= 96 (Hartley and Horne 1984; Amaro, Abuín et al. 1996; Philips and Ráb 2001). However, all studies shows an ancestor karyotype with a diploid chromosome number higher than these observed in the modern salmonids.

Robertsonian translocations are responsible for chromosome polymorphism in salmonids either at the inter or intra-individual level (Disney and Wright Jr. 1987;

Hartley 1989; Phillips and Ráb 2001). In rainbow trout, this polymorphism is more evident than in other salmonids (Hartley and Horne 1982; Colihueque, Iturra et al. 2001). Similar to rainbow trout, Robertsonian polymorphism is common in Arctic charrs. The brook trout, on the other hand, do not exhibit this type of polymorphism (Phillips and Ráb 2001).

If hybrids would inherit half of their chromosomes from each parent, i.e., 42 from brook trout and 40 or 41 from the Arctic charr, one would expect that the hybrids had 82 or 83 chromosomes (NF= 100) (Xu, You et al. 2009). However, 11 individuals with 84 chromosomes were observed. This can be related by multiple arm re-arrangement and Robertsonian fission in Arctic charr resulting in two acrocentric from one metacentric, suggesting an increase of chromosome number (1n= 42) (Danzmann, Davidson et al. 2008; Takai and Izutsu 2008). The hybrid Sf x Sa 4 karyotype was composed of lower number of chromosomes than expected (2n= 81), but had the same number of chromosome arms (NF = 100). This result is supported by the existence of more metacentric chromosomes (19) and less acrocentric chromosomes (62) than normal. Thus, the results suggest fusion of two or four acrocentric chromosomes that resulted in one or two metacentric chromosomes, respectively, as previously reported for *S. alpinus* (Hartley 1989; Pomianowski et al. 2012).

The existence of hybrids with 2n = 82 (NF= 99-100) (5 individuals) suggests that the parent (*S. alpinus)* had a diploid chromosome number of 81 and produce gametes with 40 and 41 chromosome, while the presence of hybrids with 2n = 83 (NF= 99-100) (10 individuals) suggests that *S. alpinus* could have 2n = 81 or 82 chromosomes. In addition, the variation in the number of chromosomes observed here may be related by large scattering in the technique of cell suspension leading to loss of some metaphases and chromosomes of individuals (Earley 1975; Gjedrem, Eggum et al. 1977).

The elimination of chromosomes can also be responsible for the observed changes in the chromosomes number. Chromosome elimination is a very common mechanism acting on hybrids, particularly in fishes (Arai 1984; Nakai, Kubota et al. 1995; Fujiwara, Abe et al. 1997; Iwamatsu, Kobayashi et al. 2003; Sakai, Konno et al. 2007; Xu, You et al. 2009; Kojima, Kojima et al. 2010), insects (Nicklas 1960; Tomkiel 2000) and plants (Linde-Laursen and Bothmer 1988). In Fujiwara, Abe et al. (1997) was observed that in inviable masu salmon (Ms) x rainbow trout (Rb) hybrids, the Rb chromosomes were preferentially eliminated through chromosome loss or deletion

during the early embryogenesis. Uniparental chromosome elimination can account for the unviability of the hybrids, and may be caused by incompatibility between the maternal cytoplasm and paternal genome as observed in some salmonids (Arai 1984; Fujiwara, Abe et al. 1997) and medaka hybrids (Sakai, Konno et al. 2007). The chromosome elimination hypothesis also suggests that some individuals may be aneuploid, which is observed in the hybrid progenies and may be associated with hybrid unviability at the embryonic level (Arai 1984; Babiak, Dobosz et al. 2002; Xu, You et al. 2009).

Despite of some phylogenetic proximity (Frolov 1995) brook trout (*S. fontinalis*) and Arctic charr (*S. alpinus*) differ in the number of chromosomes, their hybrid progenies are generally viable and the percentage of hybrid progenies with decreased fertility or even sterility has been observed to reach about 20-30%. According to Ma and Yamazaki (1986), such sterility may be related to the non-occurrence of synapsis between two haploid chromosomes of parental origin as a result of the lack of homologous chromosomes between species. The unpaired chromosome number can cause sterility in hybrids what has been observed. The male mule is well known case of hybrid sterility that appears as a result of an unpaired chromosome number (32 from the horse, + 31 from the donkey = 63 in the mule) (Trujillo, Stenius et al. 1962). In the ictalurid catfishes, the number of chromosome arms, and the distribution of chromosome relative sizes may have disturb meiotic division and end up with production of aneuploid gametes and/or leading sterility of F_1 hybrids (Zhang and Tiersch 1997). Functional triploid hybrids of grass carp x bighead carp or common carp x rohu, mrigal and catla can also found to be sterile (Bartley, Rana et al. 2001). Sterility in hybrids may be beneficial for the animal production. For instance, sterility induced in land animals such as bulls, pigs or poultry can increase productivity and improve meat quality (Piferrer, Beaumont et al. 2009). In aquaculture, the sterility may be very useful because may reduce energy costs of reproduction when there is no development of the gonads and improve survival rate, growth rate and meat quality of fishes as in rainbow trout and others salmonids (Zhang and Tiersch 1997; Arai 2001). On the other hand, sterile hybrids reduce the probability of transfer or change the gene pools in the wild populations (Donaldson 1996; Zhang and Tiersch 1997). For instance, sterility may be induced by triploidiziation – well know case of triploid rainbow trout production where females are sterile and males produce few and mostly aneuploid gametes (Devlin and Nagahama 2002). Polyploidy consists in numerical change in the whole set of

chromosomes. Physical or chemical shock applied during meiosis II or first cleavage can suppress cell division while allowing chromosomal division, producing triploids (Leggatt and Iwama 2003; Piferrer et al 2009). In the charrs case, hybridization between brook trout and Arctic charr followed by the triploidization may provide sterile population, very useful for aquaculture.

Only three charr hybrids from the present study were females. All others are males (12 individuals) or intersex individuals (9 hybrids). The low number of gonadal females and high intersex fish among the studied hybrids may suggest that hybrid females had problems with gonadal differentiation and their gonadal development was disturbed. Although one may hypothesize that intersexuality can be related to differences in male and female gonadal tissue proportions, and/or appearance of mosaic form from few aberrant cells, the basis to this phenomenon remains unclear and deserves further investigation (Kinnison, Unwin et al 2000). Environmental contamination may also be the cause of intersex in the wild salmonids (Kinnison, Unwin et al. 2000). Zebrafish intersex starts by developing ovaries that subsequently degenerates creating intersex gonads, which end up forming a normal testis. Some of the abnormality in the gonadal development like intersex may have occurred during early stages of testicular and ovarian tissue formation (*Oncorhynchus keta)* and functional production of eggs and sperm (*Salmo trutta*) (Devlin and Nagahama 2002). According to Devlin and Nagahama (2002) the relative number and strength of genetic sex-determining located in both the sex chromosomes and autosomes reflects in the development of male and female gonads. Both parental charr species of the sparctics show genetic sex determination but probably their chromosomes are at different stages of morphological differentiation. Thus, incompatibility between sex chromosomes or genes involved in the sex differentiation that derived from brook trout females and the Arctic charr males may affect gonadal development in the hybrids.

As we analyzed karyotypes of the hybrids using simple Giemsa staining, other techniques could be very useful for this kind of studies, especially those that enable identification of sex chromosomes in both parental species. Chromosome rearrangements could have been detected when FISH with telomeric probe applied (Phillips and Reed 1996). C-banding analyses are technique that could be very useful to detect regions rich in heterochromatin, which are good cytogenetic markers. Q- banding could also be important in identifying polymorphisms involving satellites and centromeres of specific chromosomes (Hartley 1991). Fluorescence In Situ

Hybridization (FISH) can help us to determine homology of specific chromosomes and chromosomes arms of brook trout, Arctic charr and their hybrids.

In conclusion, we believe that the Robertsonian translocations in the parental species (*S. alpinus*) along with chromosome elimination are the causes of variation in the number of chromosomes in the *Salvelinus fontinalis* x *Salvelinus alpinus* hybrids. However, it is still unclear what triggered that some of the hybrids showed intersexual gonads. We propose that different stages of sex chromosome differentiation in both *Salvelinus* species, or maybe potential elimination of the sex chromosomes, their inactivation or even conflict between these chromosomes may disturb gonadal differentiation in these hybrids (Phillips, Matsuoka et al. 2002; Pomianowski et al. 2012).

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