HUNGARIAN UNIVERSITY OF AGRICULTURE AND LIFE SCIENCES

GENETIC ANALYSIS OF HUNGARIAN BROWN TROUT POPULATIONS AND DEVELPOMENT OF A MARKER-ASSISTED BREEDING SYSTEM

Thesis of doctoral dissertation

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Gödöllő

2021

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1. HISTORY OF THE WORK, OBJECTIVES

The global capture sector is characterized by declining catches, fluctuations and overfishing, thus the globally growing demand for fish consumption is promoted the steady increase of aquaculture production, which requires improvements in farming and husbandry technology. Besides of traditional breeding methods, the importance of marker-assisted breeding system increased by the development of molecular genetic methods and the exploration of various DNA markers.

Salmonids are one of the most studied freshwater fish species in aquaculture, which can be explained by the economic importance of each species and its high population-level diversity within species. Due to the delicacy and lack of fishbones in their flesh, and the relatively good technological tolerance of the species, methods of culturing and breeding were soon developed. Nowadays, the most significant fish species produced under intensive conditions are salmonids. The brown trout *(Salmo trutta m. fario)* is a native European salmonid that is widespread worldwide and has great economic importance. The species is a resident of oxygen-rich and cold freshwaters. Currently its importance in nature conservation and recreation, thus the species is mainly cultured for introducing small streams. Hungary has about 1,200 kilometers of freshwater which can be suitable for brown trout, but their presence is limited only to colder, mountain streams, due to lack of eligible spawning area and self-sustaining stocks. In Hungary, the largest quantity of native brown trout is cultured in Lillafüred, as well as a smaller quantity for conversation purposes is farmed in Szilvásvárad.

Molecular studies of the brown trout have revealed the high genetic diversity of the species and identified five evolutionary lineages of mitochondrial DNA caused by geographical isolation and adaptation to local conditions: Atlantic, Danubian, Mediterranean, Adriatic and Marble. Each lineage is associated with different drainage areas and they have formed during the colonization processes following the last glaciation period. Due to the hydrogeography of Hungary, wild populations should theoretically belong to the Danubian lineage, however, despite intensive research on European populations, this has not been verified by genetic studies on Hungarian wild stocks. In Europe, anthropogenic hybridization of lineages, the mixing of foreign genes without isolation, has been described in several countries, which is increasingly threatening the survival of native populations. Reducing anthropogenic processes and protecting local small populations has become increasingly important across Europe. A number of initiatives have been launched to conserve geographically distinctive lines by eliminating the introduction of non-native lineages and marker-assisted breeding using local lineage.

Based on these, I aimed to assess the population genetic status of the Hungarian brown trout populations, and to investigate the genetic composition and evolutionary lineage of the stocks. For this purpose, I selected mitochondrial and nuclear PCR-RFLPs and microsatellite markers, which are widely used in the analysis of brown trout, and which are also suitable for distinguishing the main evolutionary lineages and characterizing the genetic composition of the populations. For further characterization of the populations, study a sex determination gene conserved in salmonids is also planned. Finally, my aim was to develop a marker-assisted breeding system to increase the proportion of native lineages in domestic broodstocks based on the results of analysed markers.

2. MATERIALS AND METHODS

2.1. Locations and sampling

Altogether 888 brown trout were sampled from two brown trout broodstocks as well as from six wild streams (Bán, Jósva, Kemence, Apátkút, Kölöntés, Bittva) in Hungary and from one wild population in Serbia **(Table 1)**. In 2011, 401 individuals were sampled from the Lillafüred population. In 2013, further 243 breeder candidates were also sampled in the Lillafüred broodstock for this study, these fish originated from outside of the hatchery for refreshing the stock and were kept separately from the older breeders stock mentioned above. The stocking and fishing history of natural populations is variable or unknown. As a control, a historically pure and geographically isolated, small Danubian population was sampled from the Panjica stream in Dobrače, Serbia in the Danubian drainage. Individuals from the Lillafüred broodstocks were tagged with PIT (Passive Integrated Transponder) for later identification. A clip of the anal fin of each fish was taken and stored in 96% at -20°C until DNA isolation.

Table 1: Investigated farmed and wild populations of brown trout in Hungary. N: number of sampled and analysed individuals by microsatellites and PCR-RFLPs, N_{seq}: number of analysed samples of mitochondrial control region sequencing

Population	ID	Type	N	N_{seq}	Year of sampling	Coordinates of sampling locations
Lillafüred 1.	**LF1**	Hatchery	401	41	2011	N48°07’03” E20°34’07”
Lillafüred 2.	**LF2**	Hatchery	243	26	2013	N48°07’03” E20°34’07”
Szilvásvárad	**SZV**	Hatchery	75	27	2014	N48°04’55” E20°24’25”
Bán	**BA**	Wild	25	12	2012	N48°08’35” E20°28’21”
Jósva	**JO**	Wild	33	16	2012	N48°28’56” E20°32’49”
Kemence	**KE**	Wild	24	20	2012	N47°59’32” E18°57’36”
Apátkúti	**AK**	Wild	50	28	2013	N47°44’53” E18°59’40”
Kölöntés	**KO**	Wild	14	14	2013	N47°22’17” E18°59’40”
Bittva	**BI**	Wild	9	9	2014	N47°13’19” E17°33’21”
Panjica (Serbia)	**SRB**	Wild	14	12	2014	N43°39’34” E20°04’20”
Total	**10**		**888**	**205**		

2.2. DNA isolation

Genomic DNA was isolated from fin clips using the E.Z.N.A. Tissue DNA Kit (Omega Bio-tek) according to the protocol of the manufacturer. The quantity of isolated DNA was determined by spectrophotometry and the quality by agarose gel electrophoresis (1% agarose, 1×TBE buffer, 0.5 µg/ml ethidium bromide). For polymerase chain reaction the concentration of each sample was adjusted to 50 ng/µl based on the concentrations measured by the nanophotometer.

2.3. Analysis of the sex ratio of brown trout populations

The sex ratio of brown trout populations was investigated using a sex-specific marker (sdY) on the Y chromosome of salmonids (Yano et al., 2013). To optimize the marker and as a positive control, 60 additional market-sized individuals at the Lillafüred site were dissected. The primers used for the sdY marker are listed in **Table 2**. The reaction was performed in a 25 µl final volume in a 2720 Thermo cycler machine (Applied Biosystems) with 1×PCR buffer $(NH_4)_2SO_4$ (ThermoFisher Scientific), 200 µM dNTP mix (ThermoFisher Scientific), 132 µM forward and reverse primer, 1.5 mM $MgCl_2$ (ThermoFisher Scientific), 0.04 U/µl Taq polymerase (ThermoFisher Scientific) and 100 ng template DNA. The reaction temperature profile was 94 ºC for 3 min, followed by 30 s at 94 ºC, 30 s at 60 ºC and 30 s at 72 ºC for 40 cycles, and finally 72 ºC for 5 min. The reaction result was determined by agarose gel electrophoresis (1% agarose, 1×TBE buffer, 0.5 µg/ml ethidium bromide).

2.4. Investigation of brown trout populations in the mitochondrial and nuclear genome

Three PCR-RFLP markers were used to distinguish brown trout lineages. In addition, I measured the genetic diversity of the populations using five additional trout-specific microsatellite markers. The used markers are summarised in **Table 2**.

Table 2: Applied genetic markers to analyses of brown trout populations. For PCR-RFLP markers restriction enzymes, and for microsatellite markers primer-linked dyes are indicated in the table.

Marker	Type	Primers	Enzyme/ Dye	References
CR mtDNA	Mitochondrial PCR-RFLP and sequencing	F: 28RIBa: CACCCTTAACTCCCAAAGCTAAG R: HN20: GTGTTATGCTTTAGTTAAGC	Fnu4HI	Bernatchez & Danzmann 1993; Sušnik et al. 2001
LDH	Nuclear PCR-RFLP	F: GGCAGCCTCTTCCTCAAAACGCCCAA R: CAACCTGCTCTCTCCCTCCTGCTGACGAA	BslI	McMeel et al. 2001
SL	Nuclear PCR-RFLP	F: TGGCCCGTTGAATCCATATAAAG R: ACTGTGAAACACTAAGCTCTCCA	MspI	Ford 1998

Marker	Type	Primers	Enzyme/ Dye	References
BFRO002	Microsatellite	F: ATGTTTTTGACTGCACTATGTATTG R: GGAGATAAGAGTCAACGAGGC	NED	Sušnik et al. 1997
OMM1064	Microsatellite	F: AGAATGCTACTGGTGGCTGTATTGTGA R: TCTGAAAGACAGGTGGATGGTTCC	VIC	Rexroad et al. 2002
Ssa408uos	Microsatellite	F: AATGGATTACGGGTACGTTAGACA R: CTCTTGTGCAGGTTCTTCATCTGT	PET	Carney et al. 2000
SsoSL417	Microsatellite	F: TTGTTCAGTGTATATGTGTCCCAT R: GATCTTCACTGCCACCTTATGACC	FAM	Slettan et al. 1995
SsoSL438	Microsatellite	F: GACAACACACAACCAAGGCAC R: TTATGCTAGGTCTTTATGCATTGT	FAM	Slettan et al. 1996
sdY	Sex-specific (Y chromosome)	F: ATGGCTGACAGAGAGGCCAGAATCCAA R: CTTAAAACCACTCCACCCTCCAT	-	Yano et al. 2013

2.4.1. PCR-RFLP markers

One locus in the control region of mitochondrial DNA (CR mtDNA) and two additional loci in the nuclear genome (lactic acid dehydrogenase (LDH) and somatolactin (SL) genes) were examined by PCR-RFLP **(Table 2)**. Reactions were performed in 25 µl final volume in a 2720 Thermo cycler machine: 1×PCR buffer $(NH_4)_2SO_4$, 200 µM dNTP mix, 264 µM forward and reverse primers, 1.5 mM $MgCl_2$ and 0.04 U/µl Taq polymerase. 200 ng of template DNA was added to the reaction for the detection of LDH and SL and 250 ng for the CR mtDNA. For the SL locus, an additional 0.5 mM $MgCl_2$ solution, and for the CR mtDNA locus, 625 ng/ml BSA (ThermoFisher Scientific) solution were added. The temperature profile of the reactions was: 3 min at 94 ºC, followed by 1 min at 94 ºC, 1 min at 50 ºC (CR mtDNA and SL) or 58 ºC (LDH), and 1 min at 72 ºC for 40 cycles, and finally 5 min at 72 ºC. The results of the reactions were determined by agarose gel electrophoresis (1% agarose, 1×TBE buffer, 0.5 µg/ml ethidium bromide). The PCR products were digested with restriction endonuclease as in **Table 2** at 37 (CR mtDNA, SL) or 55 ºC (LDH) for 8 h in a final volume of 10 µl with the following composition: 5 µl PCR product, 1× enzyme buffer, 2 U enzyme. The results of the reactions were determined by agarose gel electrophoresis (2% agarose, 1× TBE buffer, 0,5 µg/ml ethidium bromide). The different haplotypes and alleles were distinguish based on the nucleotide polymorphisms in Danubian and Atlantic lineages **(Table 3)**. For the CR mtDNA and the LDH markers, digestion with restriction endonuclease results two fragments in Atlantic samples, while no cut represents in Danubian variants. For the SL marker, two fragments are observed in the Atlantic lineage after endonuclease digestion, while three fragments are observed in the Danubian samples. Since the

LDH and SL loci are located in the nuclear genome, they have two alleles, in the case of maternally inherited mitochondrial DNA, only one allele is found at a given locus.

Table 3: Evolutionary lineage clustering of applied PCR-RFLP and microsatellite marker alleles based on literature data.

Marker	Danubian allele size (bp)	Atlantic allele size (bp)	Other allele size (bp)	References
CR mtDNS	1088	654 és 434	-	Bernatchez & Danzmann 1993; Sušnik et al. 2001
LDH	428	353 és 75	-	McMeel et al. 2001; Marić et al. 2010
SL	396, 317 és 189	713 és 189	-	Ford 1998; Snoj et al. 2010
BFRO002	122-126	116-118	120	Sušnik et al. 1997; Jug et al. 2005
OMM1064	173-282	172-261	178, 204	Rexroad et al., 2002; Lerceteau-Köhler & Weiss 2006; Bogataj 2010
Ssa408uos	231-282	211-227	219, 233, 235, 239, 243, 255, 258	Carney et al., 2000; Lerceteau-Köhler & Weiss 2006; Bogataj 2010
SsoSL417	158, 169, 179, 194	173, 175, 190, 191, 197	177, 181, 184	Slettan et al., 1995; Lerceteau-Köhler & Weiss 2006; Bogataj 2010
SsoSL438	97, 99, 105	95, 103, 108, 110	101, 106	Slettan et al., 1996; Lerceteau-Köhler & Weiss 2006; Bogataj 2010

2.4.2. Sequencing of mitochondrial genome

In addition to PCR-RFLP analysis of the control region of mitochondrial DNA, the populations were also compered based on the analysis of CR mtDNA haplotypes. Randomly chosen samples (at least 10 %) of each sample group were analysed. Purification of CR mtDNA PCR products were performed using 0.5 U Sap and 1.1 U ExoI enzyme. The sequencing reaction was performed on the purified PCR product using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) in a final volume of 10 µl: 1×BigDye buffer, 132 µM 28RIBa or HN20 primer, 1 µl BigDye, 1-7 µl PCR product. Reaction temperature profile: 28× 96°C for 10 s, 50°C for 5 s and 60°C for 4 min. The product was precipitated using sodium acetate-ethanol process, and after removal of the supernatant, the precipitate was dried, and the samples were dissolved in HiDi formamide (Applied Biosystems). After incubation for at least 10 h, the mixture was denatured at 96°C for 6 min in a Thermal Cycler. Capillary gel electrophoresis was performed on a 3130 Genetic Analyzer (Applied Biosystems) using POP7 polymer (Applied Biosystems). To ensure the reliability of the results, bi-directional sequencing was performed multiple times.

2.4.3. Microsatellite markers

To further characterise the populations, five microsatellite markers commonly used in salmonids were selected **(Table 2)**. For the analysis of BFRO002, OMM1064, Ssa408uos and SsoSL417 loci, the reaction was performed in a final volume of 25 µl with the following composition: 1×Taq-polymerase buffer containing $(NH_4)_2SO_4$, 264 µM forward and reverse primers, 200 µM dNTP mix, 1.5 mM $MgCl_2$ and 0.04 U/µl Taq-polymerase and 200 ng template DNA. The thermal profile of the reactions was 3 min at 94°C, followed by 35× 94°C for 45 s, 57°C for 60 s (BFRO002, SsoSL417 loci) or 90 s (OMM1064, Ssa408uos loci) and finally 72°C for 60 s, with cycles of 5 min at 72°C. The SsoSL438 loci were also tested in 25 µl reaction volume: 1× Taq polymerase buffer containing $(NH_4)_2SO_4$, 132 µM forward and reverse primers, 200 µM dNTP mix, 2 mM $MgCl_2$ and 0.06 U/µl Taq polymerase and 100 ng template DNA. The reaction temperature profile was 3 min at 94°C, followed by 35× 94°C for 40 s, 50°C for 40 s and 72°C for 40 s, with cycles of 5 min at 72°C.

For accurate measurement of fragment lengths, primers with fluorescent labelling were used. In the case of the SsoSL438 locus, the reverse primer used in the PCR reaction was provided with 5' FAM fluorescent labeling (direct labeled primer). For the BFRO002, OMM1064, Ssa408uos and SsoSL417 loci, the forward primer was extended with a 17 bp long non-species-specific fragment (tail: 5'-ATTACCGCGGCTGCTGG-3'). For the PCR reaction, a third primer (tail primer), complementary to the tail, was added at the 5' end, labelled with a fluorescent dye (PET, FAM, VIC or NED) at 264 µM (Shimizu et al., 2002). Both methods result labelled PCR products at the 5’ end of the fragments. The results of the reactions were verified by agarose gel electrophoresis (2% agarose, 1×TBE buffer, 0.5 µg/ml ethidium bromide), and successful reactions were prepared for fragment analysis using the following solution: 0.5 µl FAM/NED/PET or VIC fluorescent dye-labelled PCR product, 0.2 µl GeneScan 500 LIZ molecular weight marker (Applied Biosystems) and 9.8 µl HiDi Formamide. The mixture was denatured at 94°C for 6 min in a Thermal Cycler. Fragment analysis was performed on an ABI 3130 machine using POP7 polymer. The raw data were processed using GENEMAPPER SOFTWARE VER. 4.0 (Applied Biosystems), and accurate size of fragments were prepared in EXCEL sheet (Microsoft).

2.4.4. Data analysis

2.4.4.1. Mitochondrial genome variability and network analysis

The sequences of CR mtDNA were visualized, aligned and analysed by the MEGA 5 software (Tamura et al., 2011) using Danubian (AY185568) and Atlantic (AY185577) reference sequences from NCBI database. Evolutionary divergence between the haplotypes was estimated with Tamura-3 parameter model (Tamura, 1992). The haplotype (Hd) and nucleotide diversity (π) of

the control region per populations were analysed in DnaSP 5.10.01 (Librado & Rozas, 2009). A haplotype network was built using the median joining algorithm in the program NETWORK 4.1.1.2 (Bandelt et al., 1999).

2.4.4.2. Population genetics of nuclear genome

The population genetic analysis of the stocks was performed using of the allele sizes of the PCR-RFLP and microsatellite loci. In case of nuclear markers, the mean number of alleles (Nma), effective allele number (Neff), observed (Ho) and expected (He) heterozygosity per locus and per population were calculated using GeneAlEX 6.5 (Peakall & Smouse, 2012). In case of the microsatellite loci, the private alleles of each populations and hierarchical analysis of molecular variance between populations (AMOVA) were analysed by the same software. Allelic richness (Ar), F_{IS} (inbreeding coefficient, proportion of the variance within population) and pairwise F_{ST} (fixation index, proportion of the variance between populations) values and tests for standard deviations from Hardy-Weinberg expectations (HWE) were calculated in FSTAT 2.9.3.2 (Goudet, 2001) The significance level of multiple comparisons was estimated using a serial Bonferroni correction (Rice, 1989).

The polymorphic information content (PIC) of microsatellite loci was determined using the EXCEL extension MICROSATELLITE TOOLKIT VER. 3.1.1 (Park, 2001). The presence of a null allele (the probability of an allele dropout or genotyping error), was estimated using MICRO-CHECKER (Van Oosterhout et al., 2004). The Neighbor-Joining tree was prepared using software POPULATIONS (Langella, 2002) based on Da-distance (Nei et al., 1983), and then the phylogenetic tree was constructed using the FIGTREE VER. 1.3. (Rambaut, 2009). To explore the possible internal substructures of the populations software STRUCTURE (Pritchard et al., 2000) was used. The possible cluster distributions between K=1 and K=12 was investigated. The most probable number of genetic clusters (K) was determined by probability analysis of each K value using STRUCTURE Harvester (Earl & vonHoldt, 2012; Evanno et al., 2005). Also based on microsatellite allele results, we plotted principal component analysis (PCA) of genetic variance between populations using R environment adegnet 2.0.1 package (Jombart et al., 2008).

2.5. Development a marker-assisted breeding system and analysis of F1 generation

In order to enrich the Danubian lineages in the Lillafüred broodstock, a scoring system was created based on the allele sizes of PCR-RFLP and microsatellite loci. Based on literature data, the alleles of microsatellite loci was classified into Danubian, Atlantic and other groups **(Table 3)** (Sušnik et al. 1997; Jug et al. 2005; Lerceteau-Köhler & Weiss 2006; Bogataj 2010). In case of the OMM1064 locus, alleles in the overlapping size range were assigned to one lineage based on previous studies, or to novel/other alleles in other cases. Individuals were scored based on their

Danubian allele proportion: one point for the cellular alleles, one point for rare alleles (occurring up to five times in the whole population), and only for the female individuals had ten points for Danubian mitochondrial DNA. The scores of the individuals were assigned to their unique identifier **(Figure 1)**, thus during breeding easily can be determine that how many Danubian alleles a given individual has using PIT reader. We used this method to create a new generation in November 2014 and then tested 31 individuals from the F1 generation for the same loci.

Figure 1: The scoring system based on the markers used and the score assigned to the individual identifier (Genetic azonosító) in the marker-assisted breeding system

CR mtDNS	SL		LDH		BFRO 002		OMM 1064		Ssa 408uos		SsoSL 417		SsoSL 438		Ivar	Dunai MT	Ritka allélok száma	Dunai allélok száma	Összes nukleáris dunai allél	Ikrás dunai mtDNA	Genetikai azonosító
100	100	200	100	200	116	116	197	228	235	247	177	190	103	108	2	0	0	5	5	0	L260_5
100	100	200	100	200	116	120	172	228	205	250	175	177	103	105		0	0	5	5	0	L262 _5
100	100	100	100	200	116	120	232	267	235	247	173	175	97	105		0	0	4	4	0	L263 _4
100	100	100	100	100	116	116	247	247	235	235	181	177	97	105		0	0	2	2	0	L264 _2
100	100	100	100	100	116	120	197	228	219	227	177	177	103	105	2	0	0	2	2	0	L265 _2
100	100	100	100	100	116	116	176	178	250	235	190	181	97	105	1	0	0	1	1	0	L266 _1
100	100	100	100	100	116	116	172	197	211	219	173	181	105	105	1	0	0	0	0	0	L267 _0
100	100	200	100	100	120	120	172	227	227	247	177	177	103	106		0	0	6	6	0	L268 _6
100	100	200	100	100	116	120	172	208	258	258	175	190	103	103		0	0	5	5	0	L269 _5
100	100	100	200	200	116	120	197	228	219	227	175	177	103	105		0	0	4	4	0	L27_4
100	100	100	100	100	116	116	267	288	215	235	173	181	105	105		0	0	1	1	0	L270_1
100	100	200	100	100	116	116	232	247	223	227	175	175	103	105		0	0	3	3	0	L271 _3
100	100	100	200	200	116	116	208	267	223	235	188	190	105	108	2	0	0	5	5	0	L272 _5
100	100	100	200	200	116	116	243	267	205	235	177	188	97	99		0	0	4	4	0	L273 _4
100	100	200	200	200	113	120	208	261	247	258	173	173	97	103	1	0	0	7	7	0	L274 _7
100	100	100	100	100	116	120	172	172	219	235	181	188	97	99	2	0	0	1	1	0	L275 _1
100	100	200	200	200	116	116	197	232	239	247	177	190	103	105		0	0	5	5	0	L276_5
100	100	200	200	200	116	120	232	243	205	258	175	175	103	103		0	0	7	7	0	L277 _7
100	100	200	100	200	116	116	172	179	205	235	188	188	99	103		0	0	4	4	0	L278 _4
100	100	200	100	200	116	116	208	267	235	247	181	188	97	99	1	0	0	5	5	0	L279 _5
100	100	100	100	200	116	116	172	261	239	247	177	181	103	103	2	0	0	4	4	0	L28_4
100	100	100	100	200	*110*	113	164	176	219	239	173	177	97	105	1	0	1	1	2	0	L280_2
100	100	200	100	100	116	120	*158*	172	219	235	179	188	97	99	1	0	1	3	4	0	L281_4
100	100	200	200	200	120	120	178	208	205	258	175	177	103	103		0	0	8	8	0	L282 _8
100	100	100	100	200	116	116	208	261	239	247	173	175	103	105		0	0	4	4	0	L283 _4
100	100	100	100	100	116	120	232	261	219	227	175	177	103	105	2	0	0	2	2	0	L284 _2
100	100	200	100	200	116	116	182	227	227	247	175	190	103	105		0	0	5	5	0	L285 _5
100	100	100	100	200	116	120	168	182	205	247	173	181	97	103	1	0	0	4	4	0	L286 _4
100	100	100	100	200	116	116	172	172	219	235	181	188	97	99		0	0	1	1	0	L287 _1
100	100	200	100	200	116	116	197	208	219	250	177	177	97	105		0	0	4	4	0	L289_4

3. RESULTS

3.1. Sex ratio of brown trout populations in this study

The sdY marker was optimized to determine the sex ration in Hnngraina and Serbian brown trout populations using tissue samples from dissected fishes. Analysis was performed on all individuals (487 in total) except for the LF1 stock. The results showed that the proportion of females was around 50% in LF2 and SZV broodstocks and in Kemence stream, while the proportion of females was less than 50% in the other wild stocks **(Table 4)**.

Table 4: Proportion of the female individuals and the Danubian lineages in cultured and wild brown trout populations in Hungary based on sdY and PCR-RFLP markers. The minimum and maximum values within the populations are highlighted. N: number of samples, Nf: proportion of female individuals, CR mtDNA: proportion of individuals with Danubian mitochondrial haplotype, Nf CR-da: proportion of female individuals with Danubian mitochondrial haplotype within the population, LDH-da: proportion of individuals with Danubian LDH allele, SL-da: proportion of individuals with Danubian SL allele, n.d.: no data.

Population	N	Nf (%)	CR mtDNS (%)	Nf CR-da (%)	LDH-da (%)	SL-da (%)
LF1	401	n.d.	0,25	**0,0**	37,0	22,1
LF2	243	49,0	49,2	25,9	**9,9**	33,9
SZV	75	51,0	22,7	9,3	21,3	22,7
BA	25	26,1	8,7	4,4	31,3	**34,1**
JO	33	36,4	9,1	3,0	10,6	17,2
KE	24	50,0	4,2	**0,0**	41,7	29,2
AK	50	44,0	34,0	16,0	19,0	18,0
KO	14	35,7	**64,3**	**28,6**	**42,9**	3,6
BI	9	22,2	**0,0**	**0,0**	22,2	**0,0**
SRB	14	42,9	100,0	42,9	100,0	100,0

3.2. Distribution of the lineages in brown trout populations of this study

3.2.1. Variance of mitochondrial

PCR-RFLP analysis of the mitochondrial DNA control region confirmed the pure Danubian origin of the Serbian control population at this locus. However, in all Hungarian populations the Atlantic lineage was present, and in most Hungarian sites the Atlantic lineage was predominant (51-100%). The exception is the Kölöntés stream, where Danubian haplotype was found in 64% of the individuals. The inheritance of mitochondrial DNA is exclusively linked to the female sex, and the proportion of female individuals with Danubian haplotypes varied from 0-29% within

populations, with the highest proportion in Kölöntés stream (28.6%) and in the LF2 broodstock (25.9%), while in the Kemence and Bittva streams and in the LF1 broodstock any female individuals with Danubian mitochondrial haplotype was found **(Table 4)**.

The alignment of the 205 CR mtDNA sequences have provided 753 bp comparable sequences with 11 polymorphic sites. The sequences of nine haplotypes are accessible in Nucleotide database of National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/) with accession numbers from MG751088 to MG751096. The analysis of sequence variation has revealed nine haplotypes **(Table 5)**, six of which were assigned to the Danubian and three to the Atlantic lineage.

Table 5: Nucleotide divergence and positions between different CR mtDNA haplotypes. Da: Danubian haplotype; At: Atlantic haplotype.

Nucleotide position (bp)	46	96	135	136	289	290	430	442	443	448	450
Da1	G	A	A	G	T	C	C	A	C	T	T
Da2	.	.	T	.	.	.	.	.	.	.	.
Da3	.	.	G	.	.	.	.	.	.	.	.
Da4	.	C	G	.	.	.	.	.	.	.	.
Da5	.	.	T	.	.	.	.	.	.	.	C
Da6	.	.	G	.	.	.	.	.	.	.	C
At1	.	.	.	T	C	T	T	G	G	C	.
At2	.	.	.	T	C	T	.	G	G	C	.
At3	A	.	.	T	C	T	T	G	G	C	.

The median-joining network **(Figure 2)** indicated two haplotype groups: Atlantic and Danubian. The Atlantic group consisted of all Atlantic haplotypes and the Danubian included all Danubian haplotypes. There was no conflict between the results of mitochondrial PCR-RFLP and sequence analyses in any of the analysed individuals. One of the Danubian haplotypes (Da6 GenBank acession no. MG751095) has not been described previously and was represented in only one individual of the Kemence stream. This haplotype also belongs to the Danubian group based on the median-joining network. The size of the circles is proportional to the frequency of haplotypes within the sample set, while the length of the lines is proportional to the number of mutation steps between haplotypes. The two groups are separated by six mutation steps and the divergence between them is 0.0112. Within the Atlantic cluster, the At2 and At3 haplotypes are not directly connected, probably the At2 haplotype existed first, then the At1 and At3 variations derived from it. In the Danubian cluster, the Da1 haplotype branches off into the Da2 and Da3 variations, which then branch off into the other haplotypes. The newly found Da6 haplotype is located one mutation step away from the Da3 and Da5 haplotypes, while it is separated from the other haplotypes by two mutation steps.

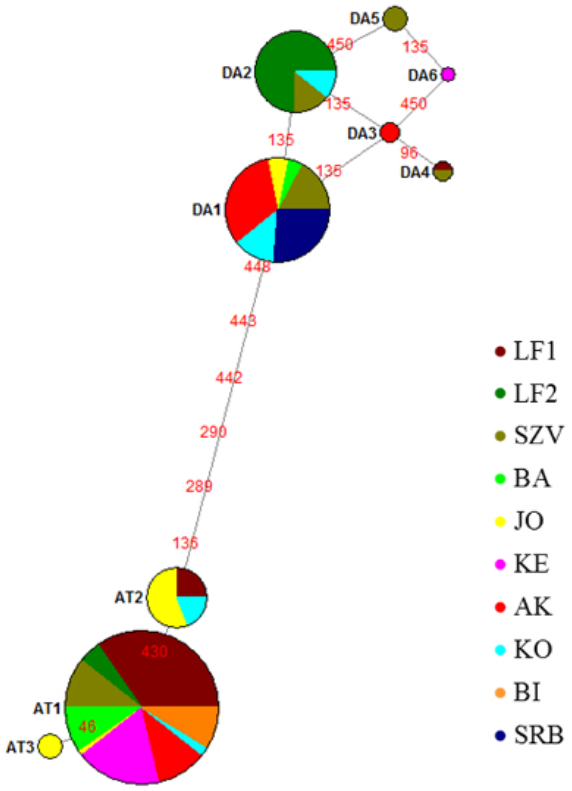


Figure 2: Median joining network analysis based on the CR mtDNA haplotype diversity of the studied brown trout populations. The red numbers indicate the nucleotide position of the mutation, the size of the circles represents the frequency of haplotypes within the sample set.

The most abundant Danubian haplotype (Da1) appeared, beside the Serbian control, in the wild populations and the Szilvásvárad broodstock. Da2 haplotype was detected in both hatcheries and Kölöntés stream. Da3 variant was only presented in the Apátkúti streams as a private haplotype with low frequency, while the Da4 and Da5 haplotypes were only detected in the cultured stocks. The most frequent Atlantic haplotype (At1) was found in all populations with the exception of the Serbian control. The At2 haplotype was identified in the LF1 broodstock and in two wild populations, while the At3 haplotype was only present in the Jósva stream. Only one haplotype was presented in the Serbian control population as well as in the Bittva streams, so the diversity within these populations is 0, while the haplotype diversity of the other populations ranges from 0.100 to 0.758. The haplotype diversity (Hd) of total sample set is 0.67 (π) and the nucleotide diversity is 0.005 **(Table 6)**. Haplotype analysis of CR mtDNA and network analysis revealed a mixture of lineages or a predominance of the Atlantic lineage in all Hungarian populations and a difference in the genetic composition between the LF1 and LF2 broodstocks.

Table 6: Number of identified CR mtDNA haplotypes in cultured and wild brown trout populations. The table shows the NCBI identifiers of the haplotypes that I identified and their earliest described, and the newly found haplotype (Da6, MG751095) is highlighted in bold.

N: sample size, Da: Danubian haplotypes; At: Atlantic haplotypes; Hd: haplotype diversity; π: nucleotide diversity

Population	*N*	Da1	Da2	Da3	Da4	Da5	**Da6**	At1	At2	At3	Hd	π
		AY185568	AY185573	AY185570	HQ848372	HQ848373	**MG751095**	EF530480	EF530476	AY185577		
LF1	*41*				1			36	4		0,224	0,001
LF2	*26*		21					5			0,323	0,003
SZV	*27*	8	4		1	3		11			0,738	0,006
BA	*12*	2						10			0,303	0,003
JO	*16*	3						1	9	3	0,650	0,004
KE	*20*						**1**	19			0,100	0,001
AK	*28*	15		2				11			0,574	0,003
KO	*14*	6	3					2	3		0,758	0,005
BI	*9*							9			0,000	0,000
SRB	*12*	12									0,000	0,000
Total	***205***	**46**	**28**	**2**	**2**	**3**	**1**	**104**	**16**	**3**	**0,670**	**0,005**

3.2.2. Variance of nuclear DNA

To investigate the nuclear genome lineages, PCR-RFLP analysis of LDH and SL loci in each population was performed. Similar to the CR mtDNA, these loci confirmed the pure Danubian origin of the Serbian control population, and I observed a predominance of the Atlantic lineage in all Hungarian populations (LDH: 57-90%, SL: 66-100%) **(Table 4 and Figure 3)**. Interestingly, no Danubian alleles were found for the SL and CR mtDNA loci in the Bittva stream, but the proportion of Danubian alleles reached 22% for the LDH locus. In the Kölöntés stream, higher proportion of Danubian alleles in the LDH (43%) and CR mtDNA (64%) loci was found, but for the SL marker the proportion of Danubian alleles was less than 4%. Remarkable differences were found in the genetic composition of the two Lillafüred broodstocks in these loci. Among the Hungarian population, any pure Danubian individuals in either the mitochondrial or the nuclear PCR-RFLP loci were found.

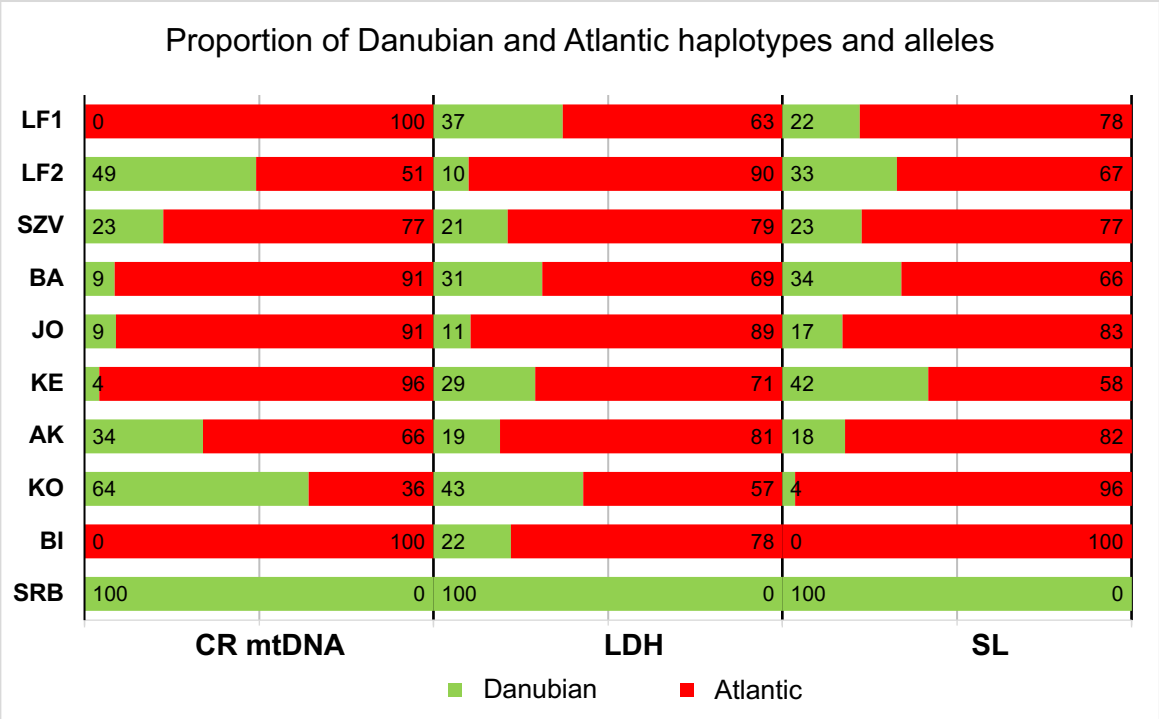


Figure 3: Proportion of Danubian (green) and Atlantic (red) alleles/haplotypes in analysed brown trout populations based on three PCR-RFLP loci

3.3. Population genetic status of brown trout populations based on the analysis of nuclear markers

3.3.1. Genetic diversity within population

Two allelic PCR-RFLP loci showed low to moderate polymorphism in the populations studied (PIC=0.07-0.37), and due to the presence of homozygous individuals in the Serbian control

population and Bittva stream, the SL and LDH loci had zero polymorphic information content (PIC). In contrast, microsatellite loci showed different levels of polymorphism. Considering the total number of alleles and the PIC per population, the microsatellite locus BFRO002 with few alleles showed moderate polymorphism (PIC=0.07-0.53), while the other microsatellite loci showed high polymorphism in all populations (average PIC=0.57-0.86). The highest PIC values (0.95) were measured at the OMM1064 locus **(Table 7)**.

The typical allele size of BFRO002 locus was 110-124 bp, OMM1064 locus 158-364 bp, Ssa408uos locus 208-309 bp, SsoSL417 locus 161-194 bp and SsoSL438 locus 99-116 bp. In general, the larger sample sizes of the population had higher locus variance. Private alleles with variable frequencies (0.001-0.332) were presented in all populations. Total of 42 private alleles were found, representing 30% of all alleles and were mainly associated with the OMM1064 and Ssa408uos loci. The highest number of private alleles (n=11) was found in two Lillafüred broodstocks, and the highest proportions were found in the isolated Bittva (6-17%) and Kölöntés (23-32%) streams **(Table 7)**.

Table 7: Private allele sizes (bp) and freqencies of microsatellite loci in each population (range 0-1, 1=100%)

Population	Locus	Allele size	Frequency
LF1	BFRO002	110	0,004
	OMM1064	158	0,001
		164	0,006
		216	0,003
		234	0,001
		239	0,001
		263	0,001
		346	0,003
	SsoSL417	169	0,001
	SsoSL438	93	0,003
		97	0,162
LF2	OMM1064	186	0,123
		225	0,009
		286	0,003
		296	0,003
		337	0,003
	Ssa408uos	213	0,002
		225	0,007
		269	0,002
		281	0,002
		285	0,002
		303	0,002
SZV	OMM1064	331	0,007
BI	OMM1064	202	0,167
	Ssa408uos	245	0,056
	SsoSL417	196	0,056
JO	OMM1064	250	0,061
		272	0,015
		277	0,030
		316	0,106
	Ssa408uos	253	0,045
		260	0,015
		273	0,015
KE	OMM1064	162	0,045
AK	OMM1064	166	0,104
		224	0,052
KO	OMM1064	174	0,321
	Ssa408uos	289	0,231
BA	OMM1064	252	0,020
	Ssa408uos	284	0,020
SRB	OMM1064	206	0,036
	Ssa408uos	309	0,250

The results of population genetic analysis of study sites are presented in **Table 8**. The mean number of alleles (Nma) in the two nuclear PCR-RFLP loci was 2 in all populations except the homozygous Serbian control. The sample size-dependent allelic richness (Ar) ranged from 1.000 to 2.000 in each population, while the allele frequency-dependent effective allele number (Neff) ranged from 1.000 to 1.825. The highest expected (He=0.459) and observed (Ho=0.708) heterozygosity values were measured in Kemence streams, where the inbreeding coefficient value (F_{IS}=-0.561) also indicated a high predominance of heterozygosity. Significant deviation from Hardy-Weinberg equilibrium ($p<0.01$) was observed in Kemence stream and LF1 broodstock, in both cases caused by heterozygote excess measured on SL locus.

The mean number of alleles ranged from 3.75 to 18.60, the allele richness values from 4.805 to 7.276 and the effective allele number from 2.118 to 7.446 in the joint analysis of microsatellite loci. For these indicators, the lowest values were observed in Bittva stream, and Serbian control population, which can be explained by the lower number of individuals. The joint analysis of microsatellite loci showed that all populations are in Hardy-Weinberg equilibrium. However, separated analysis of these loci revealed heterozygote excess in LF2 (BFRO002, F_{IS}=-0.111, $p<0.05$, Ssa408uos, F_{IS}=-0.070, $p<0.001$), and heterozygote deficiency in Jósva stream (BFRO002, F_{IS}=0.377, $p<0.05$) and LF1 (SsoSL417, F_{IS}=0.083, $p<0.01$) populations. The probability of null alleles was only shown in the LF1 population for the SsoSL417 locus.

Table 8: Summary of the population genetic analyses of two nuclear PCR-RFLP and five microsatellite (MS) loci for cultured and wild brown trout populations in Hungary. N:number of samples, Nma: mean number of alleles, Neff: effective allele number, Ar: Allelic richness, He:expected heterozigosity, Ho: observed heterozigosity, Fis: inbreeding coefficient, HWE: significant deviation from Hardy-Weinberg equilibrium, NA=not available, ns=not significant, ** $p<0.01$, *** $p<0.001$

Population	N	Marker	Nma	Neff	Ar	He	Ho	F_{is}	HWE
LF 1	401	PCR-RFLP	2	1,699	1,995	0,406	0,441	-0,088	**
		MS loci	16,20	6,917	6,901	0,772	0,783	-0,014	ns
LF 2	243	PCR-RFLP	2	1,515	1,927	0,314	0,306	0,026	ns
		MS loci	18,60	7,004	6,848	0,740	0,781	-0,055	ns
SZV	75	PCR-RFLP	2	1,523	1,992	0,345	0,373	-0,081	ns
		MS loci	10,25	6,271	7,276	0,762	0,765	-0,003	ns
BA	25	PCR-RFLP	2	1,785	2,000	0,449	0,521	-0,163	ns
		MS loci	10,50	7,145	7,102	0,812	0,884	-0,091	ns
JO	33	PCR-RFLP	2	1,316	1,944	0,241	0,277	-0,157	ns
		MS loci	12,25	7,446	6,866	0,691	0,680	0,016	ns

Population	N	Marker	Nma	Neff	Ar	He	Ho	F_{is}	HWE
KE	24	PCR-RFLP	2	1,825	2,000	0,459	0,708	-0,561	***
		MS loci	6,75	3,764	4,815	0,685	0,692	-0,010	ns
AK	50	PCR-RFLP	2	1,432	1,983	0,305	0,290	0,048	ns
		MS loci	8,25	4,841	5,613	0,676	0,671	0,007	ns
KO	14	PCR-RFLP	2	1,517	1,882	0,290	0,321	-0,114	ns
		MS loci	5,75	3,993	5,772	0,629	0,685	-0,093	ns
BI	9	PCR-RFLP	1,5	1,264	1,500	0,183	0,222	-0,231	ns
		MS loci	4,75	3,415	5,000	0,732	0,711	0,030	ns
SRB	14	PCR-RFLP	1	1,000	1,000	0,000	0,000	NA	NA
		MS loci	3,75	2,118	4,805	0,565	0,571	-0,012	ns

3.3.2. Genetic diversity among populations

In case of nuclear PCR-RFLP markers, the Serbian control population was significantly different from the other populations (F_{ST}=0.558-0.920, $p<0.001$). Moderate to high levels of significant divergence were found among Hungarian populations (F_{ST}=0.027-0.231, $p<0.05$), and Bittva, Kölöntés and Kemence streams were the most divergent populations (F_{ST}=0.116-0.231). Analysis of microsatellite loci indicated significant genetic divergence among all populations ($p<0.001$). Paired F_{ST} values indicated a moderate genetic difference between Hungarian populations (F_{ST}=0.042-0.217) and a high degree of divergence between Hungarian populations and Serbian control population (F_{ST}=0.222-0.369). The mean F_{ST} value for all populations was 0.110 ±0.039. In addition to the Serbian control, the largest differences were measured in case of Bittva and Kölöntés streams.

Based on the allele frequencies of the microsatellite loci, the Nei genetic distances (Da) between populations were determined and a phylogenetic tree of the populations (Neighbor-joining, NJ) was constructed **(Figure 4)**. The results also revealed that Serbian control population is most distinct from other populations (Da=0.537-0.682). The highest genetic distance among Hungarian populations (Da=0.582) was measured between same populations as the highest genetic differentiation was observed (Fst=0.217): between Bittva and Kölöntés streams. These two populations also showed the highest genetic distance from the other populations. In addition, the dendrogram also revealed moderate genetic distance between the wild and cultured populations (Da=0.165-0.582), similar to the F_{ST}.

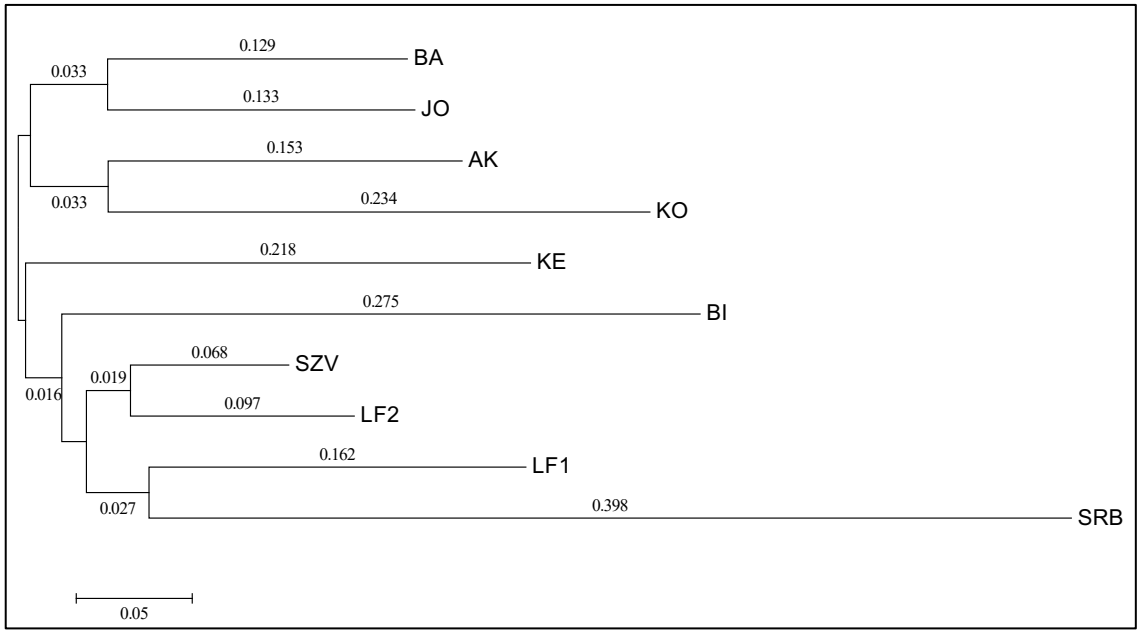


Figure 4: Neighbor-joining phylogenetic tree based on Da distances (Nei et al., 1983) calculated from allele frequencies of microsatellite loci. The numbers represent the distance from the node.

To investigate the genetic composition of the whole sample set STRUCTURE and PCA analyses were made based on microsatellite allele frequencies. STRUCTURE software determined the genetic structure of populations without geographic assignment, assuming the presence of different clusters. The analysis using STRUCTURE Harvester software did not give a clear ΔK result, revealing 2, 3 and 5 possible clusters of the sample set, i.e. samples from 10 sampling sites could be genetically classified into 2, 3 or 5 distinct groups **(Figure 5)**. The highest probability of cluster numbers was at 2 and the lowest at 5. These results reflected to the dendrogram layout based on Da distances **(Figure 4)**, but no clusters were restricted to a single population. In all cases, individuals from Lillafüred broodstocks (LF1 and LF2) belong to different clusters, and in case of clusters 3 and 5, they were also separated from the other populations. The clustering of individuals in the Szilvásvárad broodstock (SZV) showed an intermediate group between other broodstocks and wild populations. The Serbian control was most separated from the other populations in case 5 clusters, however, it has not formed a separate cluster. Analysis of whole sample set, the least variance was observed among the individuals from Apatkúti stream.

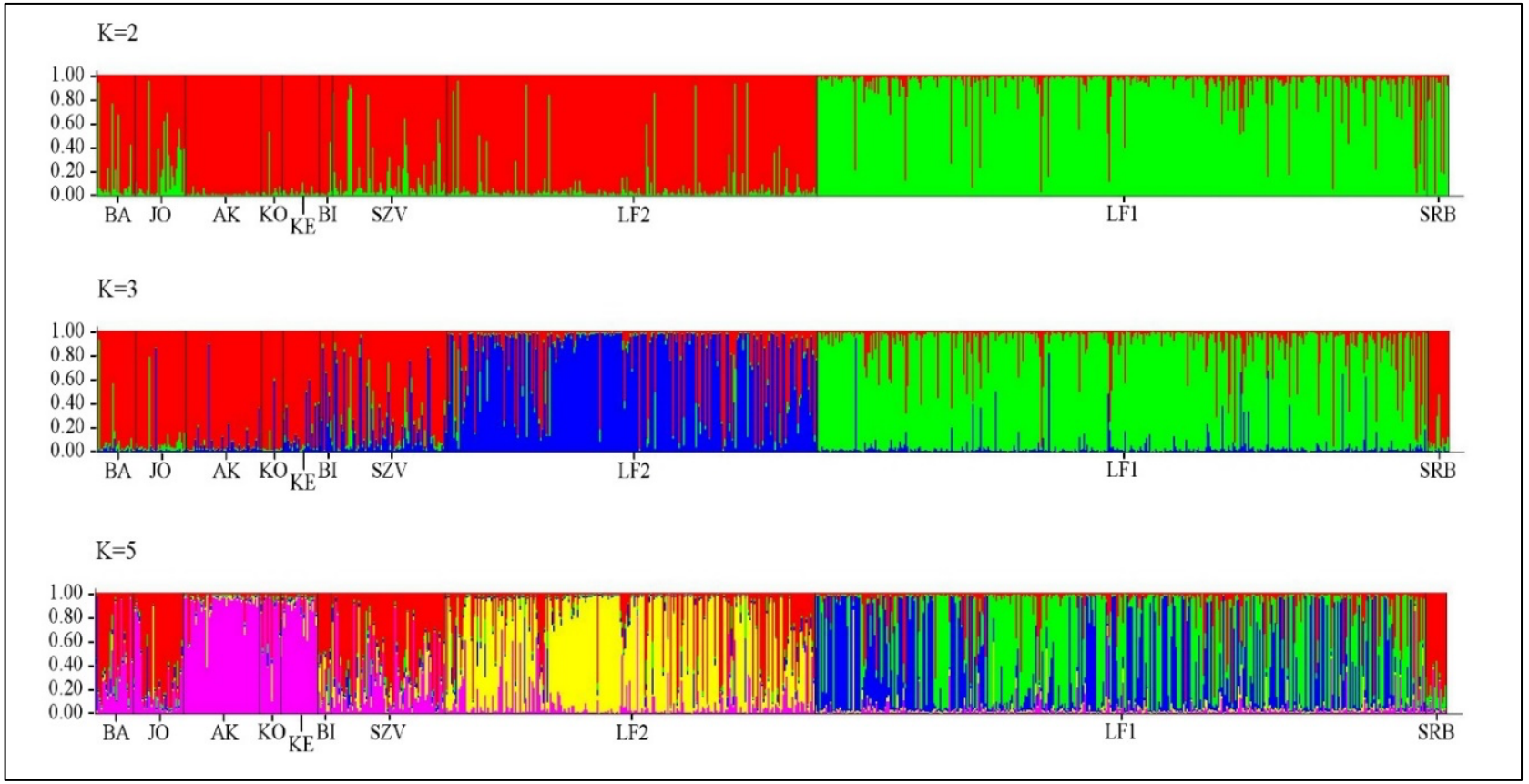
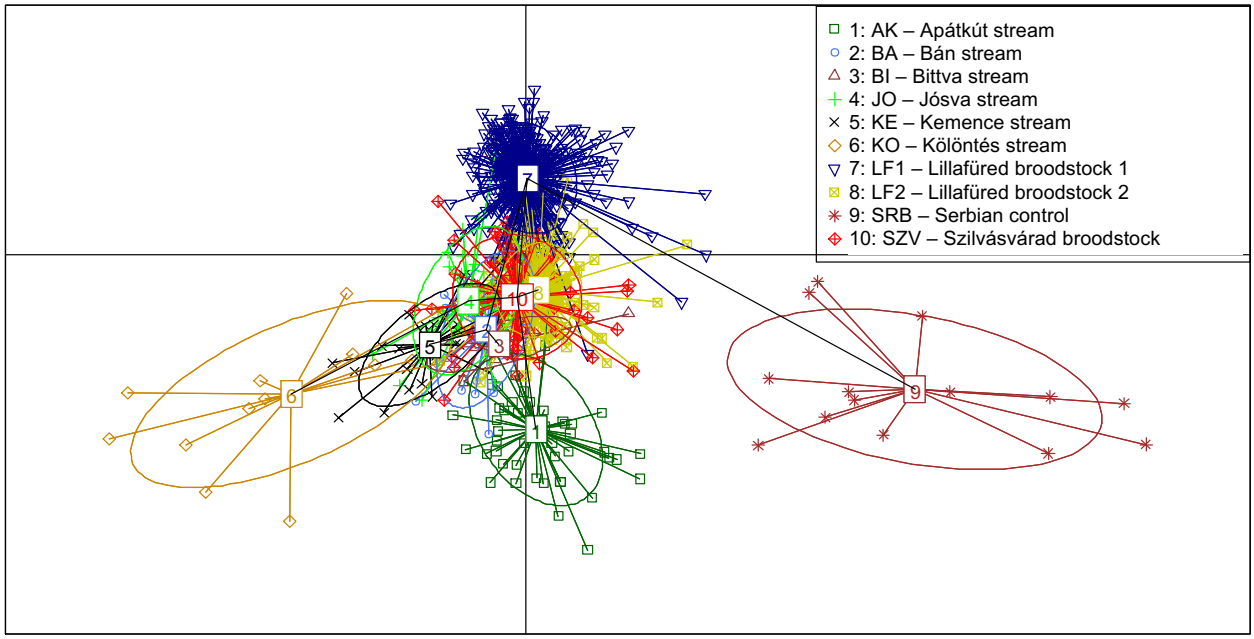


Figure 5: Bayesian individual assignment implemented using STRUCTURE for K = 2, 3 and 5 clusters without using geographical area as a prior. The y-axis represents the probability of assignment of an individual to each cluster.

Based on the genetic distances, PCA (Principal Component Analysis) analysis was performed, which showed a similar composition to the STRUCTURE analysis with more structured clustering **(Figure 6)**. The axes divide the individuals into three main clusters, one mainly consisting of the individuals from LF1 broodstock and one from the Serbian population. The third major group is mainly located along the vertical axis and is composed of overlapping individuals from other populations. As in the STRUCTURE analysis, the individuals of Apatkúti and Kölöntés streams are separated from the third cluster.



6. ábra: Principal component analysis (PCA) of the sampled brown trout individuals.

Both structural analyses suggested moderate degree of separation among populations, which was supported by the results of the AMOVA analysis. The AMOVA analysis indicated low variance among populations (10% of total variance, degree of freedom=9), while 90% of total variance was within populations (degree of freedom=1775), which mainly related to subindividual variance (81% of total variance, degree of freedom=888), and only low variance was observed between individuals (9%, degree of freedom=878).

3.4. Development of a marker-assisted breeding system

Two Lillafüred broodstocks, altogether 644 individuals, were involved in the marker-assisted breeding system. In the scoring system based on nuclear alleles, the average score of individuals was 3, with a minimum of 0 and a maximum of 9. In total, 93 female individuals with the Danube mitochondrial haplotype were found. Analysis of 31 individuals of F1 generation, produced in

November 2014, showed a large increase in the proportion of the Danubian haplotype in CR mtDNA (from 19 to 65%) and a small increase in the Danubian allele (from 27 to 35%) at SL locus, but no enrichment of Danubian alleles was observed at LDH locus (Figure 7). For the microsatellite loci, the lineage classification of alleles is not clear due to the large number of new alleles, but overall, based on the literature data a high proportion of Danubian alleles was observed at all loci in the F1 generation (e.g. SsoSL438 locus).

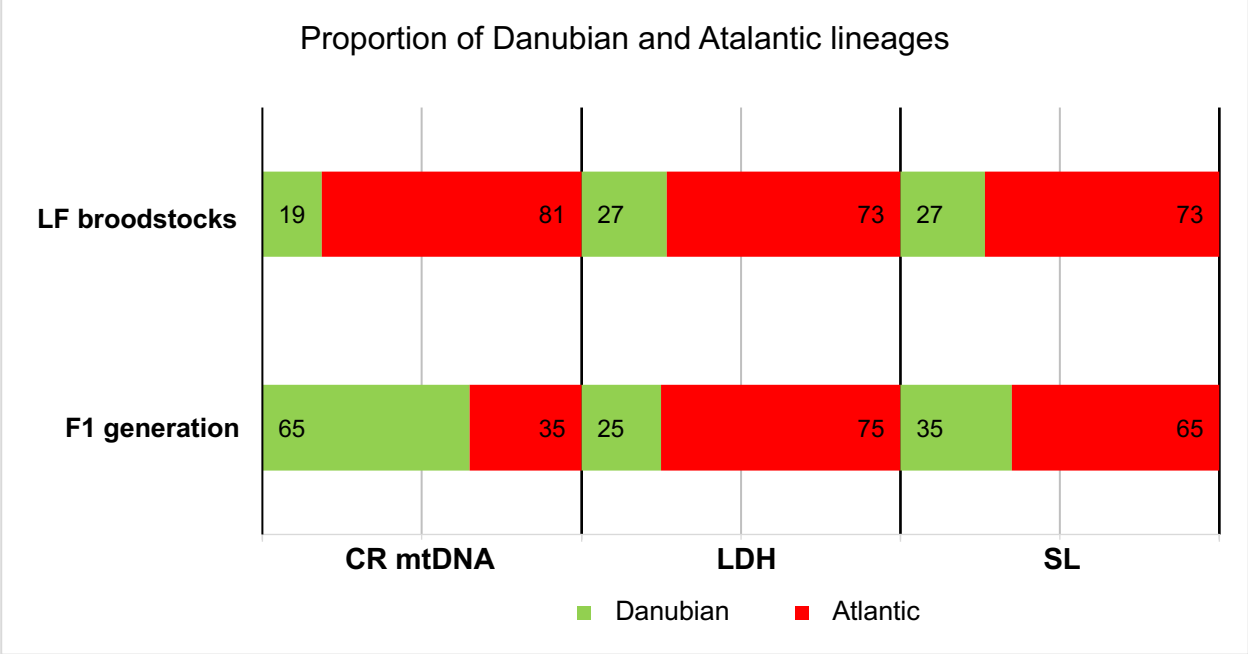


Figure 7: Proportion of brown trout with Danubian (green) and Atlantic (red) alleles/haplotypes in the Lillefüred broodstocks (n=644) and in the F1 generation (n=31) produced by markers-assisted breeding system based on the analysis of three PCR-RFLP loci

4. CONCLUSIONS AND RECOMMENDATIONS

4.1. Evolutionary lineages of brown trout populations in this study

I assessed the genetic composition of six Hungarian and one Serbian wild populations and three broodstocks of brown trout using eight DNA markers. The applicability of these markers in genetic analysis has been demonstrated by several previous studies, but the differential effect of gene drift for each marker, which may be influenced by low sample numbers, must be considered (Hansen et al., 2000; Henry & Ferguson, 1985; Horreo et al., 2015; Marić et al., 2006). The numbers of the samples were highly dependent on the sampling conditions, hydrogeographic characteristics or the amount of fish in the watercourse. For example, Bittva stream could only be sampled at three points (each 100 m long), a few kilometres apart, and the sample sizes for other populations were also relatively low, especially in Kölöntés stream and Serbian control group.

Very little information is available on the origin of the current brown trout populations in Hungary and what is known comes mostly from anecdotal information. The reason for this is that prior to World War I., these watercourses were mostly neglected by anglers and aquaculturists who preferred the more typical salmonid waters of the Carpathian mountains in today's Slovakia, Ukraine and Romania. The interest in the salmonid waters of the low mountain ranges of today's Hungary has increased following the Paris peace treaty of 1920. The Lillafüred trout farm has been founded in 1932 and the first shipment of trout eggs has arrived from Traismauer, Austria and Kláštor pod Znievom, Czechoslovakia (today Slovakia) in 1933. This broodstock was later the main supply of fish stocked into various streams in Hungary. Stocking of fish from this broodstock has been documented in the Bán and Jósva streams investigated in this study (Hoitsy, 2002). Szilvásvárad broodstock of brown trout was originated from individuals naturally occurring in the Szalajka stream, but no official data are available on its further development.

The mixed origin of the broodtstocks and genetic admixture of Atlantic and Danubian lineages in the wild brown trout populations were revealed. The sampling sites are located in the Danube basin, so I expected to find mainly alleles typical for the Danubian lineage, but a high proportion of Atlantic haplotypes and alleles in all native stocks was found. The Atlantic haplotypes found in this study have already been detected in several European populations (e.g. At1 and At2: Cortey et al., 2009; At3: Duftner et al., 2003). These are widely distributed in the Atlantic basin (e.g. also in Spain, Norway and Iceland), but also in the western part of the Danube basin and in mixed wild populations were detected, e.g. in Austria, the Czech Republic, Slovakia and Italy (Cortey et al., 2009; Duftner et al., 2003; Fruciano et al., 2014; Gratton et al., 2014; Kohout et al., 2012; Meraner et al., 2007). Most of the Danubian haplotypes observed in this study were previously described only in natural populations in Austria and Slovakia (Da1, Da2, Da3: Duftner et al., 2003; Kohout

et al., 2012), and two haplotypes (Da4 and Da5), which were only presented in Hungarian broodstocks, were also detected only in Slovakian streams before this study (Kohout et al., 2012). Haplotype Da6 has not been observed or reported previously, however it belongs to Danubian clade based on network analysis. Occurrence of this haplotype was limited to a single individual in Kemence stream, but further sampling of this population could clarify the private haplotype.

Similar to my results, introgressive hybridisation of different mitochondrial lineages of brown trout has been detected in several locations in the Danube basin. In Czech Republic and Slovakia, the predominance of the Atlantic lineage (66-95%) in the Danube basin, caused by introductions from Atlantic sources, was found in 25 natural populations and five broodstocks of 638 individuals (Kohout et al., 2012). In Austria, the genetic composition of the different brown trout populations was investigated at 117 sites with 2568 individuals: the Atlantic mitochondrial lineage dominates in the Bavarian section of the Danube (74-100%), followed by an increasing percentage of Danubian haplotypes (24-70%) downstream. The pure Danubian populations were restricted to isolated watercourses in the Alps, while in the upper Danube, north-northeast from the Alps, at the boundary of the two basins, the natural occurrence of the Atlantic lineage during the last glacial period was detected. In contrast, south-southeast from the Alps, the occurrence of Atlantic lineage is clearly the result of anthropogenic introductions and subsequent introgression (Lerceteau-Köhler et al., 2013; Schenekar et al., 2014). There is limited information on the natural occurrence of Atlantic lineage in the Danube basin, and so far it has only been described at the borders of the basins. In addition, the proportion of Atlantic lineages found in the Austrian and Slovakian parts of the Danube are similar to genetic composition of Hungarian populations, so their occurrence in Hungary can be linked to stocking. Occasionally genetic admixture was documented by Hungarian hatcheries, as their brown trout breeders originated from Hungarian streams with indigenous brown trout stocks (supposedly belonging to the Danubian lineage) as well as from Austrian, German and Danish hatcheries that held breeders of Atlantic origin. Better performance of breeders with non-native alleles in hatchery conditions would explain their presence in all broodstocks (Horreo et al., 2015). Their fingerlings were stocked into Hungarian natural watercourses from 1933 until 2004, which contributed to a hybridization of the lineages. Furthermore the angling clubs in Hungary have stocked their territories with brown trout from various sources which have caused further gene flow between the lineages.

Currently no regulations exist in Hungary on stocking of natural watercourses with non-indigenous populations beyond the level of species. Although stocking is considered an important conservation activity of autochthonous populations (Taylor, 1991), but the hybridization can cause worse fitness as well as the genetic variance of the native species (Allendorf et al., 2001). Thus, in an ideal case stocking should be carried out using only individuals originating from the local

population by supportive breeding of these fishes. This strategy is widely spread in conservation of salmonids (Berrebi et al., 2000; Verspoor et al., 2007; Horreo et al., 2008; Gil et al., 2016). Due to the admixture of various lineages in Hungarian natural streams, this ideal case is not a viable option for the investigated streams, because the populations of the investigated streams are very small and there are no sufficient numbers of matured individuals to maintain the genetic diversity. Theoretically, if a unique population showing no signs of admixture was found in Hungary, we would suggest protection of that population and if stocking was necessary, this should be carried out only using that particular population. However, we cannot recommend this policy for the currently existing wild populations as they are in a Hardy-Weinberg equilibrium and do not represent unique genetic value.

Another solution could be to enrich the Danubian lineage using of marker-associated selection, then stock these individuals. This method can preserve of the genetic background of the brown trout evolved in Hungary, although it does not enale the restoration of a pure Danubian lineage. (Baric et al., 2010; Weiss, 2005). With our breeding system developed in Lillafüred broodstocks, the proportion of Danube haplotypes and alleles in the F1 generation was successfully increased, but should be careful to maintain the stock's fitness, avoid performance degradation and inbreeding (Guimaraes et al., 2007).

4.2. Population genetic status of Hungarian brown trout stocks

Population genetic analysis of the nuclear markers showed that the studied stocks are in Hardy-Weinberg equilibrium. Only in the case of the Lillafüred broodstocks and Jósva and Kemence streams, significant differences in the number of observed heterozygotes were detected in the SL and some microsatellite loci. The reduced genetic variance in some populations may be due to inbreeding, historical bottle neck effects, or isolation (e.g. Serbian population), while the high genetic diversity may be due to gene flow. The actual population size also influences the pattern of genetic diversity. Stocks with low effective population size are expected to have lower genetic diversity due to stronger genetic drift. In addition, lower diversity may suggest that few individuals have formed the population in the recent period (Pecsenye, 2006; Freeland et al., 2012). These processes may be associated with various anthropogenic effects (e.g. artificial mixing of populations), or the presence of inbred individuals with lower performance remaining after the natural populations have been established (Ferguson, 2007; Weiss & Schmutz, 1999). The results of the sdY sex-specific marker showed equal proportion of spawners in the broodstocks and Kemence stream, while in the other watercourses the proportion of female individuals was less than 50%. It is important to note that these results may be influenced by the small population size and lower sample size.

Genetic admixture between the studied populations was also confirmed by population genetic indicators. Based on F_{ST} values, moderate to medium differences between most of the Hungarian populations were found, which may indicate anthropogenic hybridization due to spatial isolation (Vähä & Primmer, 2006). It was confirmed by AMOVA analyses too, evidenced by a low level of differentiation observed among the populations, and high level of diversity were observed within the populations.

More detailed evidence of the admixture were shown by MJ tree, STRUCTURE plot and PCA results, that implies to anthropogenic transfer of fish among populations. However, the STRUCTURE Harvester could not clearly determine the number of the genetic clusters, the colour plots of STRUCTURE showed the most possible differentiations of populations with five clusters. The genetically clustered groups are dispersed over the populations that correspond with the outcome of the Median-joining network analysis of mitochondrial haplotypes. It was confirmed by PCA analyses, that also showed five genetic groups (the SBR, AK, KO, LF1 were separated from the main cluster), but the classification of the individuals was more clear than in STRUCTURE. Because of these we recommend the usage of PCA analyses next to STRUCTURE. However, we have used only a few microsatellites, the overlaps between the most streams and Szilvásvárad broodstock is evident, only the geographically district Serbian population seems separated (as it was expected). Analysis of Hungarian populations, it is clear that genetic clusters are not related to their geographical location. Only in the case of the Jósva and Bán streams a slight geographical and genetic link was observed, but these populations were also clustered with other wild populations where genetic differences can be observed. The complex genetic composition of wild clusters may be the result of anthropogenic influences or natural migration, then the non-native individuals may have mated with individuals originally inhabiting in the watercourse and already adapted to the specific environmental conditions (Keller et al., 2011).

Despite the admixture, elements of the ancestral genetic background can be found in all native populations, as indicated by the relatively high number of private alleles. Private alleles were found in all populations, but the frequencies were low, with the highest proportion detected in the Bittva and Kölöntés streams, suggesting that isolated, small populations may be important in conserving genetic diversity, as has been shown in Norway and Romania (Linløkken et al., 2014; Popa et al., 2019). However, we could not identify non-admixed individuals. It would be possible to identify "pure" individuals by increasing the number of individuals and populations analysed (Hansen & Mensberg, 2009).

Based on my results, genetic admixture due to stocking is observed in Hungarian brown trout populations, similar to other European stocks. In addition to the loss of value of unique populations due to hybridisation with non-native fish, reduced fitness of individuals in the nature has been

observed (Muhlfeld et al., 2009; Pinter et al., 2019). From the sustainable fisheries management, I propose to consider the following aspects to culture and stock brown trout:

- Determine if stocking is necessary additionally to the existing wild population. Where possible, preference should be supportive breeding rather than additional stocking.
- Avoid transfers between different watersheds and mixing of trout of different origins.
- The fish must come from the basin or region where they are released. Hydrogeographic, local environmental conditions and the results of genetic analyses should be considered.
- Broodstocks for genetic conservation purposes should not be bred for many generations without mating with native local fish to prevent genetic drift and loss of genetic variability.
- Individuals bred for genetic conservation should be raised in conditions similar to the natural environment to reduce the impact of domestication and selection
- The introduction of highly domesticated individuals and fishes from other areas should be avoided.
- Artificial breeding techniques should be optimised to maintain the genetic diversity of populations.

5. NEW SCIENTIFIC RESULTS

Based on my research I have achieved the following new scientific results:

1. I have performed the first comprehensive genetic study of six Hungarian and one Serbain wild populations and three broodstocks of brown trout. I found that the genetic composition of the Hungarian stocks overlapped, only the Serbian population can be clearly distinguished.

2. I used eight DNA markers to distinguish the evolutionary lineages of brown trout in the study. In the Hungarian populations I found a mixture of Atlantic and Danubian lineages, while in the Serbian population I found alleles specific for the Danubian lineage only.

3. I detected a novel Danubian haplotype in the mitochondrial DNA control region among individuals of the Kemence stream.

4. I described microsatellite allele sizes and private alleles specific to the populations studied.

5. I assessed the sex ratio in the studied population using a Y chromosome-linked, sex-specific marker described in various salmonids. I found equal ratio of the two sexes in the broodstocks, whereas most of the natural streams the male sex predominated.

6. I have successfully developed a marker-assisted breeding system in Lillafüred broodstock.

6. PUBLICATIONS

6.1. Publications related to the topic of the dissertation in scientific journals

1. **Ágnes Ősz**, Ákos Horváth, György Hoitsy, Dóra Kánainé Sipos, Szilvia Keszte, Anna Júlia Sáfrány, Saša Marić, Csaba Palkó, Balázs Tóth, Béla Urbányi, Balázs Kovács (2018): The genetic status of the Hungarian brown trout populations; exploration of a blind spot on the European map of Salmo trutta studies. PeerJ 6:e5152 https://doi.org/10.7717/peerj.5152
2. **Ágnes Ősz,** Lőrinc Sándor Pongor, Danuta Szirmai, Balázs Győrffy (2017): A snapshot of 3649 web-based services published between 1994 and 2017 shows a decrease in availability after 2 years. Briefings in Bioinformatics (2017) bbx159, DOI 10.1093/bib/bbx159
3. Ákos Horváth, György Hoitsy, Balázs Kovács, Dóra Kánainé Sipos, **Ágnes Ősz**, Klavdija Bogataj, and Béla Urbányi (2014): The effect of domestication on a brown trout (Salmo trutta m fario) broodstock in Hungary. Aquaculture International (2014) 22:5–11, DOI 10.1007/s10499-013-9665-2
4. **Ősz Ágnes,** Horváth Ákos, Hoitsy György, Keszte Szilvia, Sáfrány Anna Júlia, Balogh Erna, Guti Csaba, Nagy Bálint, Lefler Kinga Katalin, Urbányi Béla, Kovács Balázs (2016): Az ivararányok fenotípusos és molekuláris genetikai vizsgálata hazai sebespisztráng-állományokban. Halászat-Tudomány, Vol. 2/2. (2016) pp. 13-17

6.2. Oral presentations related to the topic of the dissertation

1. **Ősz Á**, Kovács B, Kánainé Sipos D, Hoitsy Gy, Snoj A, Keszte Sz, Sáfrány AJ, Herzinyák Z, Kaczkó D, Palkó Cs, Urbányi B, Horváth Á (2015): Genetic background and population genetics of Hungarian brown trout populations using PCR-RFLP and microsatellite markers. 15th European Congress of Ichthyology, Porto, Portugal, 7-11. September 2015, Book of abstracts p. 127-128.
2. **Ágnes Ősz**, Dóra Kánainé Sipos, Balázs Kovács, György Hoitsy, Aleš Snoj, Klavdija Bogataj, Gergely Bernáth, Zita Herzinyák, Dániel Kaczkó, Béla Urbányi, Ákos Horváth (2014): Genetic structure study of the Hungarian brown trout populations using PCR-RFLP and microsatellite markers. Adding Value –Aquaculture Europe 2014, 14th – 17th October 2014, Donostia–San Sebastián, Spain. (Abstract 935-936. p.)
3. **Ágnes Ősz**, Dóra Kánainé Sipos, Balázs Kovács, György Hoitsy, Aleš Snoj, Klavdija Bogataj, Gergely Bernáth, Zita Herzinyák, Dániel Kaczkó, Béla Urbányi, Ákos Horváth (2014): Investigation of Hungarian brown trout populations using PCR-RFLP and microsatellite markers. Integrated Perspectives On Fish Stock Enhancement, 2014 Annual

Symposium of the Fisheries Society of The British Isles, 7th – 11th July 2014, University of Hull, United Kingdom. (Abstract 22. p.)

4. **Ősz Á**, Keszte Sz, Sáfrány A J, Tóth B , Palkó Cs, Horváth J, Balogh E, Uri Cs, Guti Cs F, Lefler K K, Urbányi B, Horváth Á, Kovács B (2016): Hazai természetes vízi sebespisztráng-állományok (Salmo trutta m. fario) genetikai vizsgálata. XXII. Ifjúsági Tudományos Fórum, Keszthely, 2016. május 26.
5. **Ágnes Ősz**, Balázs Kovács, Dóra Kánainé Sipos, György Hoitsy, Aleš Snoj, Klavdija Bogataj, Gergely Bernáth, Zita Herzinyák, Dániel Kaczkó, Béla Urbányi, Ákos Horváth (2014): Genetic investigation of Hungarian wild and domesticated brown trout populations using PCR-RFLP and microsatellite markers. Fiatal Biotechnológusok Országos Konferenciája 2014, 2014. március 7. Szeged (Kivonat 33. p.)
6. **Ősz Ágnes**, Horváth Ákos, Kovács Balázs (2013): Hazai vad és tenyésztett sebespisztráng-állományok genetikai hátterének felmérése és egy genetikai markerekre alapozott tenyésztési rendszer kialakítása. SZIE Állattenyésztés-tudományi Doktori Iskola VII. fóruma, Gödöllő, 2013. június 19.

6.3. Posters and abstracts related to the topic of the dissertation

1. **Á. Ősz**, Á. Horváth, Sz. Keszte, D. Kánainé Sipos, A. J. Sáfrány, Z. Herzinyák, D. Kaczkó, Cs. Palkó, B. Urbányi, B. Kovács (2016): The effect of hyridization on wild brown trout (Salmo trutta m. fario) populations in Hungary. Aquaculture Europe 2016, 19th – 23th September 2016, Edinburgh, United Kingdom
2. **Ősz Á.**, Kovács B., Kánainé Sipos D., Hoitsy Gy., Snoj A., Urbányi B., Horváth Á. (2015): Improvement of a marker-assisted breeding system to increase the proportion of native alleles in a Hungarian brown trout broodstock. 12th International Symposium on Genetics in Aquaculture, Santiago de Compostela, Spain, 21-27. June 2015, Book of abstracts p. 198.
3. **Ágnes Ősz**, Balázs Kovács, Dóra Kánainé Sipos, Dániel Kaczkó, Zita Herzinyák, Aleš Snoj, Klavdija Bogataj, Béla Urbányi, Ákos Horváth (2013): Origin and genetic structure of several Hungarian wild and domesticated brown trout populations based on PCR-RFLP and microsatellite markers. Water & Fish Conference, 12th-14th June 2013, Belgrade, Serbia. (Abstract 305-309. p.)
4. Ákos Horváth, Balázs Kovács, Dóra Kánainé Sipos, **Ágnes Ősz**, Gergely Bernáth, György Hoitsy (2013): A marker-assisted breeding system for brown trout. Diversification In Inland Finfish Aquaculture II (Difa II), 2013. szeptember 24-26. Vodňany, Csehország.

5. Keszte Sz, **Ősz Á**, Nagy B, Sáfrány A J, Balogh E, Uri Cs, Horváth Á, Lefler K K, Hoitsy Gy, Urbányi B, Kovács B (2016); Ivar-detektálás sebes pisztráng (Salmo trutta m. fario) állományokban molekuláris genetikai markerrel. II. Fiatal Biotechnológusok II. Országos Konferenciája, Gödöllő, 2016. március 21-22.
6. Kovács B, **Ősz Á**, Keszte Sz, Nagy B, Sáfrány A J, Balogh E, Uri Cs, Horváth Á, Lefler K K, Hoitsy Gy, Urbányi B (2015): Sebes pisztráng egyedek ivarának vizsgálata molekuláris genetikai markerrel. XXXIX. Halászati Tudományos Tanácskozás, Szarvas, 2015. május 20-21.
7. **Ősz Ágnes**, Kánainé Sipos Dóra, Horváth Ákos, Kovács Balázs, Hoitsy György, Aleš Snoj, Klavdija Bogataj, Bernáth Gergely, Herzinyák Zita, Kaczkó Dániel, Urbányi Béla (2013): Hazai vad és tenyésztett sebespisztráng-állományok genetikai vizsgálata PCR-RFLP és mikroszatellit markerek analízise alapján. XXXVII. Halászati Tudományos Tanácskozás Szarvas, 2013. május 22-23. (Kivonat 6. p.)

6.4. Other scientific publications, not published in the topic of the dissertation

1. János Tibor Fekete, **Ágnes Ősz**, Imre Pete, Gyula Richárd Nagy, Ildikó Vereczkey, Balázs Győrffy (2020): Predictive biomarkers of platinum and taxane resistance using the transcriptomic data of 1816 ovarian cancer patients. Gynecologic Oncology 156 (2020) 654–661. https://doi.org/10.1016/j.ygyno.2020.01.006
2. Ádám Nagy, **Ágnes Ősz**, Jan Budczies, Szilvia Krizsán, Gergely Szombath, Judit Demeter, Csaba Bödör, Balázs Győrffy (2019): Elevated HOX gene expression in acute myeloid leukemia is associated with NPM1 mutations and poor survival. Journal of Advanced Research 20 (2019) 105–116, https://doi.org/10.1016/j.jare.2019.05.006
3. Dóra Kánainé Sipos, Gyula Kovács, Eszter Buza, Katalin Csenki-Bakos, **Ágnes Ősz**, Uroš Ljubobratović, Réka Cserveni-Szücs, Miklós Bercsényi, István Lehoczky, Béla Urbányi, Balázs Kovács (2019): Comparative genetic analysis of natural and farmed populations of pike-perch (Sander lucioperca). Aquaculture International (2019) 27:991–1007 https://doi.org/10.1007/s10499-019-00365-7
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5. **Ágnes Ősz**, Lőrinc Sándor Pongor, Danuta Szirmai, Balázs Győrffy (2017): A snapshot of 3649 Web-based services published between 1994 and 2017 shows a decrease in availability after 2 years. Briefings in Bioinformatics (2017) bbx159, DOI 10.1093/bib/bbx159

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