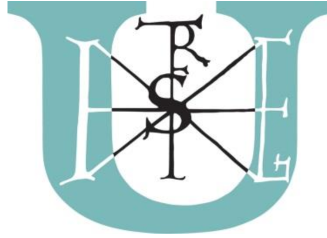

Szent István University

Isolation of microsatellite markers to investigation of fish population for genetic variability, and development of breeding

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1. THE HISTORY OF WORK, OBJECTIVES

The consumption of fish meat is increasing worldwide. This is partly due to the growing needs of the ever-growing population, and the fact, that in the Western societies more and more people are choosing fish consumption for a healthy lifestyle. It is also highly appreciated the consumption of carnivorous fish. From natural fish breeding, these increased demands can no longer be met, because catches show quite large fluctuations resulting from the significant anthropogenic influences, including pollution, habitat degradation, overfishing. Although aquaculture is a very dynamic sector in the agricultural economy, further improvements in husbandry technology and development of variants that tolerate well the technology in breeding are needed to keep up with the increased demand. In addition, it is required regular genetic monitoring of natural populations exposed to anthropogenic effects, conservation of genetic variability and regular "updating" of artificial stocks. Deep genetic knowledge of the species is required for breeding raised varieties and for conservation of the genetic values of the populations as well. It is possible with whole genome sequencing, but due to its high cost, the use of polymorphic genetic markers are still widespread.

Objectives

Our aim was to investigate predatory fish species that are becoming increasingly important in Hungary as well. The first of these is the pike-perch (*Sander lucioperca*), which is a native top-predator in Hungary, and is highly sought after by anglers and is well-liked by consumers for its delicious meat. While many research groups are working to increase the pike-perch production, there is a lack of information on the genetic background of this species and only few genetic markers available for more in-depth study of stocks. Therefore, we aimed to develop species-specific microsatellite markers and to study the genetic diversity of populations from the Danube River Basin with improved genetic markers, especially since

the variability of Central European pike-perch populations has not yet been monitored.

Perch (*Perca fluviatilis*), a member of the family *Percidae*, is also a native predator with increasing popularity. Although some species-specific microsatellites are already available for this species investigation, this toolkit is not yet sufficient to effectively examination of this species populations. Therefore, we aimed to isolate species-specific microsatellites and to determine the genetic differentiation between Hungarian and Polish populations.

Finally, the third species studied is the African catfish (*Clarias gariepinus*), which, although not native in our country, is the second largest quantity produced fish in Hungary. In Europe the Netherlands and Italy produce significant quantities, but it is also a very popular fish species in Asia and Middle-Africa. In these areas, African catfish are reared in intensive system and in pond fish hatchery in Africa. This species, which is broadly tolerant in many respects, has been the subject of countless biological research. Because there is very little information on the genetic background, we aimed to isolate as many polymorphic microsatellite markers as possible. These genetic tools can also be used to study the changing of diversity of artificially propagated and maintained stocks.

2. MATERIALS AND METHODS

2.1 Sampling

In case of pike-perch the total of 376 individuals (10 populations), in case of perch 182 (3 populations), and 32 individuals of African catfish were sampled. Table 1 summarizes the origin of the collected samples, the sampling site, the sample number, and which population type they are from. The collected fin clips from tailfins samples were stored at -20 ° C in concentrated ethanol until DNA isolation.

Table 1. Sampling places, population types and the number of samples by species.

Species	Country	Population	Type of population	Nr. of sample
Pike-perch *(Sander lucioperca)*	Germany	Upper Danube (Ge)	Wild	14
	Hungary	Kisbajcs (Kb)	Intensive recirculation system	78
		Győr (Gy)	Intensive recirculation system	21
		Lake Balaton (Ba)	Wild	60
		Dalmand (Da)	Pond hatchery	46
		Attala (At)	Pond hatchery	21
		Akasztó (Ak)	Pond hatchery	21
		Nyíregyháza (Ny)	Pond hatchery	47
	Romania	Timişoara (Ti)	Intensive recirculation system	20
		Estuary of Danube (De)	Wild	48
			All:	**376**
Perch *(Perca fluviatilis)*	Hungary	Biatorbágy (Hu-B)	Pond hatchery	80
		Dunaföldvár (Hu-D)	Pond hatchery	43
	Poland	Olstyn (Po-O)	Wild	59
			All:	**182**
African catfish *(Clarias gariepinus)*	Hungary	Szarvas and Gödöllő (Ma)	Intensive recirculation system	22
	Netherlands	Wageningen (Ho)	Intensive recirculation system	10
			All:	**32**

2.2 DNA isolation

DNA was extracted from tissue samples by phenol / chloroform isolation method (Sambrook & Russell 2001). After isolation, DNA quality was verified by agarose gel electrophoresis (1% agarose, 1x TBE buffer and 0.5 µg / ml ethidium bromide) and concentration was determined by spectrophotometer. The DNA was used for the dual purpose: 20 µg DNA for library construction and in 50 ng/µl concentration for microsatellite analysis.

2.3 Construction of libraries

Genomic libraries enriched with CA dinucleotide repeats were constructed using the modified method of Glenn and Schable (2005). The DNA required for library construction was obtained from male individuals, since males are heterogametic (Rougeot et al. 2002; Galbusera et al. 2000), which thus carry both sex chromosomes. In case of pike-perch, the male specimens for library construction were from the Dalmand population, in case of perch from the Dunaföldvár population, while in case of African catfish, the male specimens for library construction were derived from Szarvas. 20 µg of genomic DNA was digested with blunt-ended restriction enzymes (*Hae III / Alu I / Rsa I, / HpyCH4 V*). After separation of the agarose gel (2% agarose, 0.5 µg / ml ethidium bromide, 1x TBE buffer), 300-1000 bp DNA fragments were isolated from the gel using the NucleoSpin Extract II kit according to the manufacturer's recommendations. The amount of the fragment was determined by spectrophotometer, and after phosphatase treatment BoxI linker was ligated to 10 µg of fragment. The sequence of the oligonucleotides used for the BoxI linker:
BoxI forward primer: 5'-Phos-ATG TCT GAA GGT ACC ACT GCT GTC CGA AA-3'; BoxI reverse primer: 5'-CGG ACA GCA GTG GTA CCT TCA GAC AT-3'. The oligonucleotides forming the linker were hybridized in a Thermal Cycler. The adapter thus prepared was linked at 16 ° C overnight. BoxI restriction enzyme was also added to

the reaction mixture to cleave the linker-linker complexes. Linkage of the linker was confirmed by PCR starting from the linker. This was followed by enrichment, that is, the collection of DNA fragments containing tandem repeats. The fragments were hybridized with 3 'biotinylated containing $(CA)_{10}$ repeats oligonucleotides. The pulled-out single-stranded DNA fragments were become double-stranded by PCR using linker-specific primers. The PCR products were ligated into T-vector and transformed into XL10 GOLD *Escherichia coli* competent cell. Colonies were screened by blue-white selection (Ullmann et al. 1967). The size of the insert was checked by agarose gel electrophoresis (1.5% agarose and 1x TBE-buffer) following colony PCR from the M13 primer binding site encoded on the T-vector.

2.4 Sequencing and primer design

After evaluating the colony PCR reaction, products, which indicated their incorporation into the vector at >300 bp, were purified using the PCR Advanced Clean Up System. The sequences of the inserts were determined from the purified PCR products using SP6 (5' CAT ACG ATT TAG GTG ACA CTA TAG 3') and T7 (5' TAA TAC GAC TCA CTA TAG GG 3') primers with BigDye 3.1 kit by capillary gel electrophoresis. The sequences were analysed with MEGA5 software (Tamura et al. 2011). Specific primers for the flanking regions of sequences containing at least 5 dinucleotide tandem repeats were designed with Primer3Plus software (Rozen & Skaletsky 2000; Untergasser et al. 2007).

2.5 Optimization of reaction conditions

Microsatellite markers were detected by polymerase chain reaction. By optimizing the reaction conditions, the ingredients and temperature profile of the PCR reactions were precisely determined. The PCR was carried out in a final volume of 25 µl with the following ingredients: 1x Taq-polimerase buffer (containing KCl or $(NH_4)_2SO_4$), 0,132 mM-0,264 mM forward and reverse primers, 1,5-

3,00 mM $MgCl_2$, 0,2 mM dNTP, 0,04 U/µl Taq-polimerase and 150 ng template DNA. The thermocycling profile consisted of: preliminary denaturation at 95 °C for 2 min, followed by two cycles of pre-amplification (95 °C/15 s; ann.temp./1 min; 72 °C/2 min) and 35 or 45 cycles of amplification (95 °C/15 s; ann.temp./20 s; 72 °C/40 s) in case of the remaining ones. The reactions were closed by a final extension (72 °C for 5 min). The addition of DMSO (4% v/v) to amplify the following markers is also recommended: *MS 350 Cg, MS 432 Pf, MS 441 Pf, MS 455 Pf, MS 716 Pf*. At the 5' end of the amplicon, fluorescence labelling was incorporated to determine their exact length. The following ways were carried out for it: A) the forward primers with 5 'FAM fluorescent dye used in the PCR reaction (direct labelled primer); B) the forward primer was extended with a 17 bp non-species specific region (tail; 5'-ATT ACC GCG GCT GCT GG-3 ') complemented by a third, 5' end fluorescent dye (PET, FAM, VIC or NED) labelled primer (tail primer). (Shimizu et al. 2002). During the PCR reaction, the labelled oligonucleotide is built on the end of the amplicons.

2.6 Analysis of microsatellites

For each of the three species, the optimized reactions enabled PCR reactions to be carried out on a lower sample number (8-8 individuals) to check the functionality of the markers and to characterize the markers, then population genetics analysis were performed on a higher sample number. In case of pike-perch, we estimated population genetic diversity in 10 populations, for a total of 376 individuals. In case of perch, we performed a genetic comparative analysis of 2 Hungarian stocks and 1 Polish population with a total of 182 individuals. Microsatellite-based genetic characterization of African catfish was carried out in 32 individuals. The results of the reactions were checked by 2.5-3% agarose gel electrophoresis, and the successful reactions were prepared for fragment analysis by 3130 Genetic Analyser. Based on the raw data from fragment analysis, the

exact fragment length was read by GeneMapper ver. 4.0 software, the values were prepared using MS Excel for population genetics calculations.

2.7 Software used for population genetics and marker characterization

The size of the alleles determined by fragment analysis served as a basis for the calculation of population genetic indices. Expected (H_E) and observed (H_O) heterozygosity values per population and PIC (Polymorphism Information Content) per locus were determined using the Excel Microsatellite Toolkit ver. 3.1.1 (Park 2001). Allelic richness (Ar) of the markers, F_{IS} values of the populations (within-population component of genetic variance) and F_{ST} (inter-population component of genetic variance), as well as gene diversity and total allele number per locus and per population were calculated with Fstat ver. 2.9.3.2 (Goudet 1995). Deviation from Hardy-Weinberg equilibrium was determined by Genpop ver. 4.1.0 software (Rousset 2008). The average number of alleles per population and locus, average gene diversity, and F_{ST} values per population pairs are determined by Arlequin ver. 3.5 (Excoffier et al. 2005). The determination of private alleles and PCoA analysis (Principal Coordinate Analysis) were performed by GenAlEx ver. 6.502 (Peakall & Smouse 2012; Smouse et al. 2015). To determine Nei's genetic distance (Nei et al. 1983), Populations ver. 1.2.32 (Langella 2002), in the case of pike-perch for phylogenetic tree construction, FigTree ver. 1.3.1 (Rambaut 2009), and for the perch, Mega7 ver. 7.0.14 (Kumar et al. 2016) software was used. Based on the results of microsatellite analysis of the individuals, the structure of the examined stocks (without using population information) were determined byStructure ver. 2.3.3 (Hubisz et al. 2009; Pritchard et al. 2000). The most probable genetic cluster number was determined by probabilistic analysis of each K-value (L'(K), L''(K) and ΔK) using Structure Harvester (Evanno et al. 2005; Earl & vonHoldt

2012). Linkage disequilibrium (LD) of the newly developed markers and used in population genetic analysis was investigated with GENETIX VER. 4.05.2 (Belkhir et al. 1999). The probability of the presence of the null allele, the large allele dropout, and genotyping errors were calculated with MICRO-CHECKER VER. 2.2.3 (van Oosterhout et al. 2004). MICROSOFT EXCEL software was used to find whether there is a relationship between sex and genotype, that is, whether there is any connection between sex and marker (in pike-perch and perch population genetic assays). Effective population size was determined using LDNE VER. 1.31 software (Waples & Do 2008).

2.8 Multiplexing the PCR reactions

Our method (PCR based detection and fragment analysis) has been further developed to reduce the time of analysis, the cost of used materials and the laboratory work. Therefore, 16 high-allele markers were selected from the newly isolated pike-perch microsatellites, which are equal in their reaction conditions (PCR ingredients and temperature profile). Four different marker sets were created, each with 4 microsatellites are well distinguished by their allele size. Different fluorescent dyes were used in every set (PET, NED, FAM, VIC), thus, samples from the same individual can be analysed together when fragment analysis was performed (the samples could be analysed by pooling). The size of the fragments compared to GeneScan 500 LIZ molecular weight marker was determined using 3130 Genetic Analyser.

3. RESULTS AND DISCUSSION

3.1 Pike-perch (*Sander lucioperca*)

3.1.1 Construction of libraries and characterisation of the newly developed markers

Two genomic libraries enriched for CA-dinucleotide repeats were constructed from the genomic DNA of the pike-perch using two restriction enzymes (*Rsa I*, *HpyCH4 V*). The sequences were placed in a gene bank database (NCBI GenBank). From these, 34 functional markers were developed. The functionality of the markers was tested on at least 8 individuals and their characteristics were determined. All 34 functional markers were found to be polymorphic, with detected alleles ranging from 3 to 20 per locus. Most alleles (20) could be detected with the *MS 260 Sl* marker, but *MS 84 Sl, MS 192 Sl, MS 412 Sl* and *MS 424 Sl* markers are also highly polymorphic.

3.1.2 Population genetic study

Seven markers from the developed markers (*MS 192 S1, MS 195 S1, MS 198 S1, MS 203 S1, MS 260 S1, MS 268 Sl* and *MS 397 S1*) were used to study genetic diversity in 10 populations – natural and artificially maintained stocks (pond farms and recirculation systems) - from the Danube River Basin (Ak: Akasztó; At: Attala; Gy: Győr; Ti: Timişoara; Kb: Kisbajcs; Ny: Nyíregyháza; Da: Dalmand; Ba: Lake Balaton; De: Estuary of Danube; Ge: Upper Danube). From selected markers *MS 192 Sl* and *MS 260 Sl* have the highest number of alleles (17; 20), mean allele number (7; 6.7) and allelic richness (10.226; 8.661), however, the total average allele number (9.14) was also high. Surprisingly, the highest average allele numbers are found in the artificially propagated Kisbajcs (Kb: 5.43) and Dalmand (Da: 4.71) stocks.

Expected (H_E) heterozygosity ranged from 0.452-0.593, and observed (H_O) heterozygosity was 0.415-0.567. Hardy-Weinberg equilibrium is only in Timişoara (Ti) and Upper Danube (Ge)

populations. The highest average gene diversity is observed in the estuary of Danube (De) and the Attala (At) populations. A heterozygote deficiency is observed in the majority of the examined populations, but there is heterozygote excess in Kisbajcs (Kb) stock (F_{IS} = -0.038).

During studying the populations we found that the examined groups also have unique characteristics. Most (4) private alleles were detected in estuary of Danube (De) population, but the Kisbajcs (Kb) and Győr (Gy) stocks have 3-3 private alleles. *MS 260 Sl* marker has 9 private alleles, and the next *MS 397 Sl* marker has only 3. The frequency of private alleles is very low in the investigated populations, however, the frequency of private alleles for *MS 260 Sl* marker is 15% in the Upper Danube (Ge) population.

The F_{ST} for all studied populations is 0.214, which reflects the fact that genetic differentiation between populations is quite high.

Nei's genetic distance (Nei's Da) was determined, and based on this results, phylogenetic tree (Neighbour Joining) was constructed to represent the relationship between populations (*Figure 1*). The highest genetic distance (Da = 0.807) was found between Upper Danube (Ge) and Győr (Gy) populations. In addition, high genetic distances were measured between Timisoara (Ti) and Upper Danube (Da = 0.758), between Kisbajcs (Kb) and Upper Danube (Da = 0.778), as well as between estuary of Danube (De) and Upper Danube (Da = 0.713) population in pairs. The smallest genetic distance was found in the Dalmand-Lake Balaton population pair (Da = 0.040), but similarly low values were found in Akasztó-Attala (Da = 0.073) and in Kisbajcs-Nyíregyháza (Da = 0.085) population pairs.

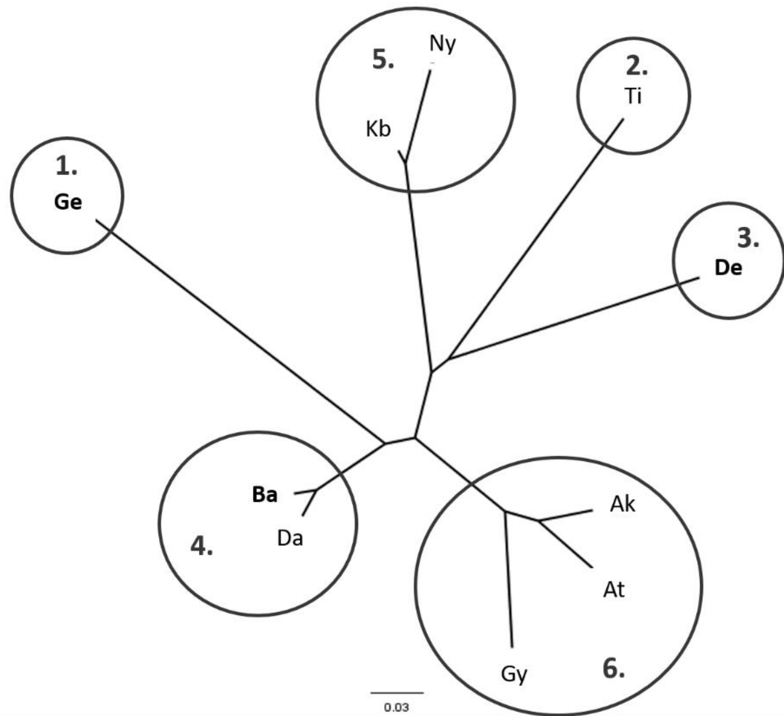

Figure 1. Dendrogram based on Nei's genetic distance using Neighbour Joining method.
Upper-Danube (Ge, 1 in the figure), Timisoara (Ti, 2 in the figure) and estuary of Danube (De, 3 in the figure) populations are distinct origin groups, while Lake Balaton (Ba) and Dalmand (Da, 4 in the figure); Kisbajcs (Kb) and Nyíregyháza (Ny, 5 in the figure); and finally Akasztó (Ak), Attala (At) and Győr (Gy, 6 in the figure) stocks form 3 other different genetic clusters. Populations marked in bold are wild (Ge, Ba and De).

Based on the genetic distance, we also performed PCoA (Principal Coordinate Analysis) analysis with the GENALEX software, as well as we used STRUCTURE VER. 2.3.3 to determine the genetic structure of the stocks assuming the presence of different number of clusters. Based on Evanno-method with the STRUCTURE HARVESTER software package calculated K = 6, as the most likely cluster number, that is, samples from the 10 sampling places can be genetically classified into 6 separate groups. No any linkage disequilibrium (LD) was found between markers used in population genetic analysis. We also examined whether the detected alleles are related to the sex, but we

did not find any relationship in this respect, because we could not find any allele that appeared in only one sex.

3.1.3 Results of multiplexing analysis

From the newly developed pike-perch markers, we selected 16 markers that are sufficiently high polymorphic, have same reaction conditions, and can be grouped into 4 separate sets based on the length of the amplified products. The functionality of the optimized multiplex reactions was tested on 4 individuals. With this method, we reduced the amount of materials used and the time required for laboratory work by nearly a quarter, and, overall, we developed a cost-effective method. The use of 10-12 polymorphic microsatellites in a population genetics analysis is usually adequate and provides a very efficient and rapid solution for large sample size analysis.

3.2 Perch (*Perca fluviatilis*)

3.2.1 Construction of libraries and characterisation of the newly developed markers

After DNS isolation two enriched libraries were constructed using 2 different restriction enzymes (*Rsa I*, *HpyCH4 V*) with CA_{10} oligonucleotide. The newly determined sequences were placed in the genbank database (NCBI GenBank). The total of 25 functional markers were developed. The optimal reaction conditions of the markers were determined, then tested and characterized in at least 8 selected individuals. The 25 functional markers were all polymorphic, with the number of detected alleles ranging from 3 to 48. Most alleles (48) could be detected with the *MS 428 Pf* marker, but the *MS 427 Pf, MS 464 Pf* and *MS 726 Pf* markers were also highly polymorphic.

3.2.2 Population genetic study

Population genetic analysis was performed with 12 markers (*MS 426 Pf, MS 427 Pf, MS 428 Pf, MS 439 Pf, MS 464 Pf, MS 467 Pf, MS 500 Pf, Ms 719 Pf, MS 725 Pf, MS 726 Pf, MS 732 Pf, MS 739 Pf*) from the newly developed markers (selected on the basis of

preliminary investigations) to compare the genetic diversity to 2 domestic pond hatcheries from Hungary (Hu-D: Dunaföldvár, Hu-B: Biatorbágy) and a natural perch population from Poland (Po-O: Olsztyn). The highest average allele number in Poland is the natural population (Po-O). For all three populations, heterozygosity is low (heterozygous deficiency has been observed) and they are significantly different from Hardy-Weinberg equilibrium, however, genetic differentiation between populations is remarkable (F_{ST}=0.247).

We searched for unique features of each population by detecting private alleles. The Hu-B population has an extremely high number of private alleles (16) by the *MS 428 Pf* marker, but even more unique is the Po-O population, for which 21 private alleles were found by analysis of the *MS 427 Pf* marker, 18 private alleles by *MS 726 Pf* marker, 14 private alleles by *MS 464 Pf* marker and 10 private alleles by *MS 725 Pf* microsatellite.

Populations were compared pairwise based on Nei's genetic distance (Da) and F_{ST} values. Nei's genetic distance is quite small (0.149) between the 2 Hungarian populations (as well as the geographical distance), but much larger between the 2 Hungarian and Polish populations (> 0.6). Same conclusion was also reached based on the F_{ST} values showing genetic differentiation. Population structure was determined by STRUCTURE analysis. The most likely genetic cluster number is K = 2, that is, populations from 3 different places can be genetically classified into 2 separate groups (based on the analysis with the above mentioned 12 polymorphic microsatellite markers). One cluster consists of the 2 Hungarian artificial stocks (Hu-D and Hu-B), the other is the wild Polish population (Po-O).

Figure 2 shows the result of PCoA (Principal Coordinate Analysis) analysis based on the genetic distance calculated by GENALEX software. The figure reflects the similarity of the Hungarian populations and the genetic isolation of the Polish population.

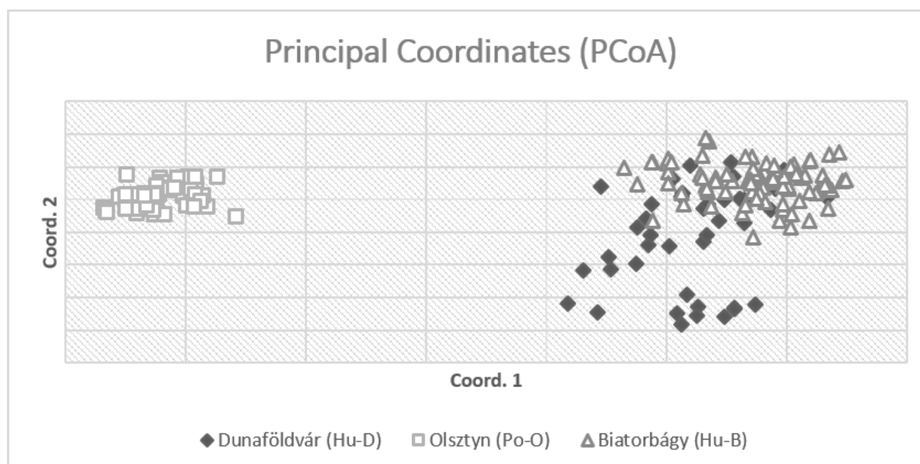

Figure 2. Result of PCoA analysis based on genetic distance calculated using GENALEX software.
Hu-D: Dunaföldvár, Po-O: Olsztyn, Hu-B: Biatorbágy.

Linkage disequilibrium was found in case of *MS 428 Pf* and *MS 439 Pf* markers. Since the *MS 439 Pf* marker has only low polymorphism, the use of this marker in subsequent analyses may be neglected. We also investigated the possibility of markers and sex linkage, but none of the loci showed linkage in any of the population.

3.3 African catfish (*Clarias gariepinus*)

3.3.1 Construction of libraries and characterisation of the newly developed markers

Four genomic libraries enriched for CA-dinucleotide repeats were constructed from the genomic DNA using four restriction enzymes (*Hae III / Rsa I / Alu I / HpyCH4 V*). Of these, a total of 55 cases were used to design PCR primers and to determine their operating conditions. Of the 55 microsatellite markers, 49 were tested for functional ability in 32 individuals with the aim of determining the functionalities and characterizing of the markers. Based on the results of this study, the 8 most polymorphic individuals were selected and the remaining 6 microsatellites tested and characterized on these individuals.

The number of alleles detected per marker ranged from 2-11. More than half (27) of the markers represented 5-6 alleles and 7 had higher allele numbers. The average total allele number was 5.12. The lowest expected heterozygosity (H_E) was 0.117 (*MS 668 Cg*), the highest was 0.793 (*MS 175 Cg*), the minimum and maximum observed heterozygosity values (H_O) ranged between 0.031 (*MS 663 Cg*) and 1,000 (*MS 3 Cg, MS 305 Cg*). The difference between the expected and observed heterozygosity is not significant at nearly half of the markers (22 markers), but for many markers (22 markers) where the difference is significant, this difference is strongly significant ($P < 0.001$ ***). The PIC (Polymorphic Information Content) values of the markers, allelic richness (Ar), and gene diversity were determined as well. The highest values were from *MS 175 Cg* marker (PIC: 0.763; Ar: 10.024; gene diversity: 0.804), and the lowest values were from *MS 308 Cg* and *MS 668 Cg* microsatellites (PIC: 0.110; Ar: 1.875; gene diversity: 0.125).

4. NEW SCIENTIFIC RESULTS

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

1. I have isolated 34 new functional polymorphic microsatellite markers from pike-perch (*Sander lucioperca*) and determined the optimal reaction conditions.

2. I have evaluated the population diversity of 10 populations from the Danube river basin with 7 of the newly isolated microsatellites from pike-perch (*MS 192 Sl, MS 195 Sl, MS 198 Sl, MS 203 Sl, MS 260 Sl, MS 268 Sl* and *MS 397 Sl*). I have proved that the majority of the studied populations differed significantly from Hardy-Weinberg equilibrium, and I also determined the population structure and genetic origin of the examined populations.

3. Sixteen of the newly isolated markers from pike-perch were included in multiplex PCR analysis using 4 sets with 4 markers each. I determined the optimal reaction conditions for multiplex PCR, which reduced the amount of laboratory work and materials used, including the cost of assay as well, by nearly a quarter.

4. I have isolated 25 new functional polymorphic microsatellite markers from perch (*Perca fluviatilis*) and determined the optimal reaction conditions.

5. I performed a comparative population genetic analysis between two Hungarian and one Polish populations with 7 of the newly isolated microsatellites from perch (*MS 426 Pf, MS 427 Pf, MS 428 Pf, MS 439 Pf, MS 464 Pf, MS 467 Pf, MS 500 Pf, MS 719 Pf, MS 725 Pf, MS 726 Pf, MS 732 Pf* and *MS 739 Pf)*. I have proved that all examined populations differ significantly from the Hardy-Weinberg equilibrium, and I determined the comparative genetic structure of the examined populations.

6. I have isolated 25 new functional polymorphic microsatellite markers from African catfish (*Clarias gariepinus*) and determined the optimal reaction conditions.

5. CONCLUSIONS AND RECOMMENDATIONS

5.1 Construction of libraries

The method of enrichment (i.e., collection of microsatellite-containing DNA fragments) was verified to be very effective compared to traditional isolation methods (Rassmann et al. 1991; Lunt et al. 1999), and similar efficiency of enrichment was achieved as the other enrichment microsatellite isolation methods. (Ostrander et al. 1992; Kandpal et al. 1994).

Although enrichment was very effective, we could not convert all the detected loci to markers, because we often find that flanking regions are unsuitable for primer design: either they are too short, or have some short repetitive sequence, or just have too high / too low GC-content.

Compared to the isolation of microsatellite and SNP markers based on next generation sequencing, the number of polymorphism-containing sequences in the case of enrichment is two orders of magnitude lower, but its cost is much lower, despite the cost of next generation sequencing. The equipment required for the implementing does not include special instruments and expensive machines (except the capillary electrophoresis system). Construction of libraries is much simpler and less labour-intensive, while primary design and marker testing need the same amount of work and cost. In addition, microsatellite markers are much more polymorphic than SNP markers, therefore, about three times fewer markers need to be tested in conservation and population genetics studies. Furthermore, with a well-designed and characterized marker set, automation of the evaluation can be achieved (Vignal et al. 2002; Helyar et al. 2011).

5.2 Pike-perch (*Sander lucioperca*)

Currently, little genetic information is available on the pike-perch population, only a few studies have been published yet (Kohlmann & Kersten 2008; Gharibkhani et al. 2009; Khurshut & Kohlmann 2009;

Han et al. 2016) despite that this species has increasing importance in the intensive fish production.

All of the developed markers have been found to be polymorphic, and we recommend markers with higher polymorphism and allelic richness for further investigation, even in closely related species (e.g. *MS 84 Sl, MS 192 Sl, MS 260 Sl, MS 412 Sl* and *MS 424 Sl*).

Of the newly developed markers with 7 markers (*MS 192 Sl, MS 195 Sl, MS 198 Sl, MS 203 Sl, MS 260 Sl, MS 268 Sl* and *MS 397 Sl*) ten populations and stocks from the Danube River basin were examined in the aspect of genetic variability, structure and relationships. We were looking for the effects of increasing stocking, intensive and pond hatchery production on populations, of which one sign was that, there was a significant difference between the expected and observed heterozygosities for most markers and for all examined populations, as well as heterozygote deficiency was detected (F_{IS} = 0.039). This is presumably due in part to overfishing and partly to the low number of specimens used in artificial reproduction. Heterozygosity could be increased by more decades of work and well thought-out colonization.

Population genetic analysis with the microsatellite marker revealed that the 10 populations showed significant genetic differentiation, but when we looked at it in pairs, we found some that were even more distinct, such as Győr (Gy), Kisbajcs (Kb), Nyíregyháza (Ny) and Timisoara (Ti) populations are significantly different from the Upper Danube (Ge) population. The population of the Upper Danube (Ge) in Germany is the most distinguished from all populations, where the frequency of private alleles is higher than by the other populations. The greatest genetic diversity was shown in the Kisbajcs (Kb) stock (intensive recirculation system), which is probably due to the fact that broodstock was repeatedly refreshed from different external sources.

This was also supported by phylogenetic tree based on the genetic distance of populations, where wild populations were located relative

to one another in accordance with their geographical locations (that is, the Lake Balaton population is located between the estuary of Danube (De) and the Upper Danube (Ge) populations). However, examined stocks of pond hatcheries and intensive recirculation systems did not fit to this approach and it is very clear which stocks are built on the same genetic basis. Investigation of the genetic structure of the populations led to the same result, the 10 populations are most likely divided into 6 genetic groups. Dalmand broodstock from the Lake Balaton, Győr broodstock from Akasztó and Attala pond hatcheries, and Kisbajcs intensive system from Nyíregyháza pond hatchery were created.

In case of pike-perch based on the above mentioned results, it appears that in the last ten years the improvement of husbandry technology (Policar et al. 2016) and breeding programs (Lappalainen et al. 2016) have not had a significant impact on the wild pike-perch populations, although due to overfishing and restocking there is a little difference from Hardy-Weinberg equilibrium. However, more intensive breeding, selection programs and restocking can lead to significant degradation of the genetic basis of the species, loss of distinct variants and the dilution or uniformity of the genetic background, as in the case of long-time breeding carp (Hulak et al. 2010), or in the case of brown trout (Ward 2006). Recognizing all this, it has become urgent to begin work on gene conservation to preserve the genetic values of the species. It can be very helpful to use of multiplex PCR to reduce costs, with which we can examine 16 microsatellite loci in the same time.

5.3 Perch (*Perca fluviatilis*)

Over the past nearly 20 years, numerous studies have been published on the genetic diversity of natural perch populations and bred populations, as well as the monitoring of anthropogenic effects. Most studies focused on populations in western (Khadher et al. 2016) and northern Europe (Olsson et al. 2011), and one study was about

perch stocks in Asia (Yang et al. 2012), but populations in Central and Eastern Europe have not yet been tested with genetic markers. Most of these studies were performed with microsatellites isolated from *Sander vitreus* and *Perca flavescens*, which are the relatives from the *Percidae* family on the American continent. However, microsatellites developed from closely related species may not be applicable in all cases and in fact often demonstrate lower polymorphism in other species (Yue et al. 2010). Microsatellites were first isolated from *P. fluviatilis* by Yang et al. (2009) and later by Pukk et al. (2014). In the present study, we would like to continue this work by developing new species-specific microsatellite markers and improving the genetic tools, and our aim was to monitor the genetic differentiation of Central European populations with this new markers at the first time.

The efficiency of the library construction used is nearly optimal, since the examined fragments carried more than 92% unique sequences, furthermore, enrichment also proved to be very effective as 93% of the resulting sequences carried typical microsatellites repeat regions.

Some of the developed markers are extremely highly polymorphic, which are strongly recommended for further analysis (*MS 428 Pf, MS 427 Pf, MS 464 Pf, MS 726 Pf*), however, the *MS 439 Pf* marker verified to be monomorphic in the examination of Hungarian stocks, hence this marker can be omitted from the further analysis.

The studied stocks differed significantly from the Hardy-Weinberg equilibrium. This is probably due to the anthropogenic effect of breeding and selection. Nevertheless, the average allele number of microsatellites was relatively high (9.611). Based on the Nei genetic distances, the pairwise F_{ST} of populations, and the analysis performed with the STRUCTURE and GENALEX software Dunaföldvár (Hu-D) and Biatorbágy (Hu-B) stocks are genetically very similar to each other (small genetic distance), they form one genetic cluster, but the Polish population (Po-O) is another genetically distinct group (larger genetic

distance). This distinction is also supported by the presence of many private alleles.

In our work, we have developed a new genetic tool system, which, in itself and in addition to the genetic markers previously described, is well suited for the detection of genetic variability in perch populations and possibly other members of the *Percidae* family. The method used by us may also be well applicable to close-related species where no high polymorphic genetic markers have yet been isolated, or the current applied tools need to be further expanded. Our work is unique, because this study is the first to carry out microsatellite-based analysis in Central European perch stocks.

5.4 African catfish (*Clarias gariepinus*)

Only 18 microsatellite markers have been described from African catfish (Galbusera et al. 1996; Volckaert & Hellemans 1999, Yue et al. unpublished data), and we have extended this genetic tool with 55 new polymorphic markers. While on the African continent the species is naturally occurring - so production is influenced by natural catches - until then all Asian and European production comes from intensive recirculation systems, this also implies that stocks may have undergone significant genetic changes. The genetic diversity of these populations could reduce, creating highly inbred populations (no natural populations are available to increase genetic variability in these areas). By determining genetic diversity with genetic markers, we can confirm or negate this degradation, and by using genetic markers, the propagation is carried out with that individuals, who are sufficiently polymorphic to increase the genetic diversity of the population.

6. PUBLICATIONS

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

Kánainé Sipos Dóra, Bakos Katalin, Urbányi Béla, Kovács Balázs (2010): Genetikai marker fejlesztése ragadozó halfajok vizsgálatához. XVI. ITF, 25th March 2010, Keszthely (ISBN: 978-963-9639-36-2)

Dóra Kánainé Sipos, Gyula Kovács, Eszter Buza, Katalin Csenki-Bakos, Ágnes Ősz, Uros 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: 1-17. DOI: 10.1007/s10499-019-00365-7.

Dóra Kánainé Sipos, Katalin Bakos, Ágnes Ősz, Árpád Hegyi, Tamás Müller, Béla Urbányi. Balázs Kovács (2019) Development and characterization of 49 novel microsatellite markers in the African catfish, *Clarias gariepinus* (Burchell, 1822). Molecular Biology Reports https://doi.org/10.1007/s11033-019-05062-5

6.2 Oral presentations related to the topic of the dissertation

Kánainé Sipos Dóra; Bakos Katalin; Müller Tamás; Urbányi Béla; Kovács Balázs (2011) Mikroszatellit markerek fejlesztése ragadozó halak vizsgálatához. Oral presentation on „Doktoranduszok Kaposvári Workshopja” conference, 8th of June 2011. Kaposvár

Kánainé Sipos Dóra, Bakos Katalin, Müller Tamás, Urbányi Béla, Kovács Balázs (2011) Ragadozó halfajok genetikai variabilitás vizsgálatának megalapozása „III. Gödöllői Állattenyésztési Tudományos Napok”, oral presentation, 14-15th of October 2011. Gödöllő

Kánainé Sipos Dóra, Bakos Katalin, Szücs Réka, Bercsényi Miklós, Urbányi Béla, Kovács Balázs (2012) A süllő (*Sander lucioperca*) populációgenetikai vizsgálata mikroszatellit markerekkel. Oral presentation on “XXXVI. Halászati Tudományos Tanácskozás” conference. 23-24th of May 2012, Szarvas

Dora Kanaine Sipos, Katalin Bakos, Reka Szucs,Tamas Muller, Miklos Bercsenyi, Bela Urbanyi, Balazs Kovacs (2012): A population

genetic study of the pike-perch (*Sander lucioperca L.*) with new microsatellite markers. Oral presentation on “AQUA 2012”. 1-5th of September, 2012, Prague, Czech Republic

6.3 Posters and abstracts related to the topic of the dissertation

Kánainé Sipos Dóra, Bakos Katalin, Urbányi Béla, Kovács Balázs (2010): Genetikai markerek növekvő jelentőségű ragadozó halfajokból. Poster presentation on “XXXIV. Halászati Tudományos Tanácskozás” conference, 12-13th of May, Szarvas

Kánainé Sipos Dóra (2010): Ragadozó halfajok genetikai vizsgálatának megalapozása. Poster presentation on “Kárpát-medencei Doktoranduszok Nemzetközi Konferenciája” (TUDOC) conference, 27-28th of May 2010, Gödöllő

Kánainé S. D., K. Bakos, T. Müller, B. Urbányi, B. Kovács (2010): Microsatellite marker isolation from carnivorous fishes Poster on Aquaculture Europe 2010, 5-8th October 2010, Porto, Portugalia

Kánainé Sipos Dóra, Bakos Katalin, Bősze Bernadett, Müller Tamás, Urbányi Béla, Kovács Balázs (2011) Mikroszatellit markerek ragadozó halak vizsgálatához. Poster presentation on “XXXV. Halászati Tudományos Tanácskozás” conference, 25-26th of May 2011, Szarvas (abstract: p.:38.)

S. D. Kánainé, K. Bakos, T. Müller, B. Urbányi, B. Kovács (2011) Microsatellite markers for newly cultured carnivorous fish in Hungary. Poster on the Genomics in Aquaculture 2011 International Symposium 14th-17th September 2011, Heraklion, Crete, Greece

D. Kánainé Sipos, K. Bakos, T. Müller, B. Urbányi, B. Kovács (2012): Isolation new, specific genetic markers from the African catfish (*Clarias gariepinus*), pike-perch (*Sander lucioperca*) and perch (*Perca fluviatilis*). Poster on 75th Anniversary of Albert Szent-Györgyi’s Nobel Prize Award. 22-25th March, 2012, Szeged, Hungay

Dóra Kánainé Sipos, Katalin Bakos, Réka Szücs, Tamás Müller, Miklós Bercsényi, Béla Urbányi, Balázs Kovács (2012): New microsatellite markers and population genetic studies of carnivorous fishes. Poster presentation on Domestication in Finfish Aquaculture 23-25th October, 2012, Olsztyn, Poland

Dóra Kánainé Sipos, Uri Csilla, Katalin Bakos, Ágnes Ősz, Máté Péli, Daniel Żarski, Zoltán Bokor, Lászlo Kotrik, István Ittzés, Béla Urbányi, Balázs Kovács (2013) Marker devolopement and population genetic studies of pearch (*Perca fluviatilis*). Poster presentation on

Diversification In Inland Finfish Aquaculture II 24-26th September, 2013, Vodňany, Chech Republic p.82.

Kánainé S. Dóra, Guti Csaba, Keszte Szilvia, Lehoczky István, Balogh Réka, Urbányi Béla, Kovács Balázs (2017) Süllő (*Sander lucioperca*) mikroszatellit markerek alkalmazásának tovább fejlesztése: idő- és költséghatékonyság növelése. Poster presentation on "XLI. Halászati Tudományos Tanácskozás" conference, 14-15th of June 2017, Szarvas

Kánainé S. Dóra, Guti Csaba, Keszte Szilvia, Lehoczky István, Balogh Réka, UrbányiBéla, Kovács Balázs (2017) Új multiplex mikroszatellit vizsgálati szett *Sander lucioperca* állományok genetikai analíziséhez. Poster presentation on "VI. Gödöllői Állattenyésztési Tudományos Nap" conference 24th of November 2017, Gödöllő

6.4 Book chapters

Kovács Balázs, Bakos Katalin és Kánainé Sipos Dóra: A harcsafajok molekuláris biológiája és genetikája. In: Horváth László, Urbányi Béla és Horváth Ákos (eds.) A harcsa (*Silurus glanis*) biológiája és tenyésztése. Gödöllő: Szent István Egyetemi Kiadó, 2011. pp 61-78. (ISBN: 978-963-269-266-1)

Kovács Balázs, Bakos Katalin és Kánainé Sipos Dóra A süllő genetikája. In: Horváth László, Urbányi Béla és Horváth Ákos (eds.) A süllő (*Sander lucioperca*) biológiája és tenyésztése (Second, revised edition). Sztárstúdió Bt., 2013. pp 55-62. (ISBN: 978-963-269-353-8)

6.5 Other scientific publications, not published in the topic of the dissertation

Katalin Bakos, Róbert Kovács, Ádám Staszny, Dóra Kánainé Sipos, Béla Urbány, Ferenc Müller, Zsolt Csenki, Balázs Kovács (2013): Developmental toxicity and estrogenic potency of zearalenone in zebrafish (*Danio rerio*). AQUATIC TOXICOLOGY (136–137):13–21.

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