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Development and application of molecular methods for the study of fish species of community and fisheries

Theses of doctoral (PhD) dissertation

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1. ANTECEDENTS AND OBJECTIVES OF THE WORK

1.1. Introduction

The world's population has grown from 5.3 billion to 7.9 billion since 1990. With the growing population, people's needs for more food and land has also increased. Industry and agriculture, in order to keep up with the growing demands, have acquired more and more land and put them into production, until now non-renewable resources seem to be running out. The results of this process are now well known to everyone. The increase in global CO2 and other greenhouse gas emissions, the climate change, the extinction of species, and the loss of biodiversity are no longer unknown concepts. In addition to environmental movements, major world organizations have also tracked change and developed various environmental strategies and action plans to conserve biodiversity.

Sustainable fishing contributes 0.1% of global GDP annually. In less developed countries, where fishing activity is essential, this value may be higher. On 14 February 2020, a global agreement was signed against illegal fishing to reduce drastic overfishing, which is contributing to the deterioration of the ecological status of our waters. Currently, fishing levels are above sustainable levels in more than a third of the fishing areas. In order to properly manage our resources, while helping to conserve biodiversity, it is essential to know all aspects of it, including genetic diversity. We still don't know enough about Hungarian freshwater fish populations, especially about genetic background of species that are traditionally non-economic importance, although in recent decades we have witnessed a number of events in Hungary that have had a drastic effect on fish fauna. Think of the cyanide pollution on the Tisza or the red mud disaster affecting the Marcal. With the development of molecular methods and the emergence of genetic markers, we have the appropriate tools to assess the genetic diversity of Hungarian fish populations, thereby mapping the current status of stocks and the effects of past events on individual populations. These studies are particularly important in the case of native and community-important species, with declining populations over time, such as the sichel (*Pelecus cultratus*) and the invasive species what is gaining more and more area and displace our native species, such as crucian carp (*Carassius gibelio*).

Recent decades have shown, that both against something (such as silver carp, introduced to suppress algal blooms and then become uncontrollable), and for something (eg. the introduction of brown bullhead into our waters for economic gain), intervening in the natural system is only possible with responsible decisions and sufficient background knowledge. Molecular genetic research, to conserve biodiversity and conserve our genetic resources contributes to this.

1.2. Objectives

1. Adaptation of microsatellite markers from closely related species for population genetic studies in the case of volga pikeperch (*Sander volgensis*) and sichel (*Pelecus cultratus*).

2. Evaluation of the applicability of mitochondrial genomic markers (Cytochrome b, Control region, Cytochrome oxidase 1) for phylogenetic and taxonomic studies in the case of invasive crucian carp (*Carassius gibelio*).

3. Investigation of the genetic diversity of non-native, invasive crucian carp populations in Lake Balaton and its catchment areas.

4. Investigation of the genetic diversity of the native volga pikeperch population in the larger waters of Hungary.

2. MATERIAL AND METHODS

2.1. Sampling and DNA isolation

During the sampling, a piece of tissue with a size of 1-2 cm2 was collected from the tail fin from each individual of the 3 fish species (sichel, volga pikeperch, crucian carp). For volga pikeperch 118 samples were collected from 3 populations. We have 72 samples from Lake Balaton, 34 samples from the Danube, and 12 samples from the Tisza and Holt-Körös units. In the case of sichel, the 128 samples were collected from Lake Balaton (n = 54), Fertő (n = 23) and the Polish Vistula Lagoon. In the case of crucian carp, 132 samples were used, from which 29 came from Siófok, 17 from Kányavár, 18 from Ingó, 19 from Őszödi-berek, 19 from Siófok-Török lakes, and 30 from Hőgyész fishing lake. Animals were anesthetized with 2-phenoxyethanol in all cases prior to tissue collection. After sampling, the tissue pieces were transferred to 1.5 ml centrifuge tubes, containing absolute ethanol and stored at -20 °C until further use. DNA isolation was performed using the E.Z.N.A tissue DNA isolation kit (Omega Biotek, Norcross, GA, USA) according to the manufacturer's protocol.

2.2. Microsatellite studies

From the 50 ng / µl DNA, the sequence detail to be examined was amplified by polymerase chain reaction (PCR). The microsatellite markers used for volga pikeperch (Kohlmann & Kersten, 2008; Kánainé et al., 2019a) were derived from the perch (Sander lucioperca) species. The primers used were fluorescently labelled oligonucleotides. Each primer carried a 17 bp (5'-ATTACCGCGGCTGCTGG-3') universal extension (tail). This is complementary to a third primer, labelled at the 5' end of the fluorescent primer. When added to the reaction, the fluorescent primer, together with the tail's forward and the reverse, is integrated into the amplified amplicon (Shuelke, 2000).

The PCR protocol was optimized either by changing the PCR components (10X (NH4)2SO4, MgCl2, dNTP (2.5 mM), oligonucleotide primers (6.6 uM), Taq (5U/ µl)) or by changing the thermal profile of the reaction (application temperature, number of cycles). For volga pikeperch, the protocol we used was a two-step PCR protocol. The number of replicates per first cycle was 2 for each marker (MSL-1, MS 701, MS 704, MS 404, MS 395). In the second step, the number of cycles was 25 for the MSL-1 marker and 45 for MS 701, MS 704, MS 404, MS 395 markers. For sichel, step 2 was not required in the PCR. During the 40 cycles of amplification, the annealing temperature was 56°C.

After optimising the PCR protocols, the next step was capillary electrophoresis. During sample preparation, 0.1 µl of molecular weight marker (GeneScan 500 LIZ (Applied Biosystems, USA)) and 9.9 µl of Hi-Di formamide were added to the PCR products. In each case, the reaction mixture for capillary gel electrophoresis was measured in a final volume of 10 µl. The diluted product was denatured at 94 °C for 6 min in a ProFlex PCR apparatus (Thermo Fisher Scientific, Waltham, Massachusetts, USA) prior to capillary gel electrophoresis. The capillary gel electrophoresis was performed using NanoPOP7 (Applied Biosystem) polymer on a 3130 Genetic Analyzer (Applied Biosystems, USA). at the Institute of Aquaculture and Environmental Safety of the Hungarian University of Agricultural and Life Sciences through a 50 cm long capillary tube.

2.3. Mitochondrial analyses

For mitochondrial genome analysis, PCR was performed using the same reaction mixture of 25 µl final volume for all three species (stonefly, silvery minnow, chard) and all three markers (D-loop, Citochrome b, Citochrome oxidase 1), consisting of $1\times(NH4)_2SO_4$ buffer (Fermentas; Thermo Fisher Scientific, Waltham, MA, USA), 2000 µM dNTP mix, 250 nM primer, 1. 5 mM $MgCl_2$ and 100 ng template DNA, and 1 U Taq polymerase enzyme (Fermentas). PCR purification was performed using the GenElute PCR purification kit from Sigma Aldrich manufacturer (Merck, Darmstadt, Germany), according to the manufacturer's protocol.

For sequence reads, I prepared PCR products purified from excess reaction components in three steps. First, the samples were re-run on 1.5% agarose gel to check the success of the purification steps and the quality of the products, and then a sequencing PCR was performed on the purified PCR product using a BigDye Terminator V3.1 sequencing kit (Applied Biosystems, Foster City, CA, USA). The sequencing reaction was pooled in a 10 µl final volume containing 2 µl BigDye, 2.5 µl 1X BigDye buffer and 2 µl 6.6, µm primer in addition to the purified PCR product. Sequencing reactions were performed in both reverse and forward directions for all samples of all three species. The samples were then dried under a sterile booth to remove any residual alcohol from the tissue surface, dissolved in 20 µl of HiDi formamide per sample and incubated at 4 °C for at least one night. After incubation, the samples were denatured at 94 °C for 6 minutes before the sequencing.

Base pair determinations were performed on the first samples using four 50 cm capillaries on the 3130 Genetic Analyzer (Applied Biosystem, USA), also used for capillary gel electrophoresis before 2019, and then on a newer type of 3500 Genetic Analyzer (Applied Biosytem, USA) using eight 50 cm capillaries.

2.4. Data analysis using population genetics software

For the microsatellite markers, the raw data reads by the Genetic Analyzer were visualized using GENEMAPPER SOFTWARE VER. 4.0 (Applied Biosystem), and the resulting curves were evaluated and the allele sizes were recorded in an EXCEL (Microsoft) spreadsheet. Population genetic analyses were performed using GENALEX VER 6.5 (Peakall & Smouse, 2012) and GENEPOP VER 4.7 (Rousset, 2008). Cluster numbers characterizing the population structure were determined using STRUCTURE VER 2.3.4 (Earl & vonHoldt, 2012) and the results were evaluated using the STRUCTURE HARVESTER program (Evanno et al., 2005). Discriminant analysis of principal components (DAPC) was performed using the software package "adagenet" in R 4.1.0 (Jombart, 2008).

For mitochondrial markers, raw sequence data were imported into MEGA X software (Kumar et al., 2018). After reading the chromatograms, the sequences were trimmed and aligned in Mega software using CLUSTALW (Higgins & Sharp, 1988), and the alignment was imported into DNASP6 (Rozas & Rozas, 1995) software in FASTA format and population genetic analyses were performed. Haplotypes identified from polymorphic base sites were checked using the National Center for Biotechnology Information (NCBI) BLAST

(Basic Local Aligment Search Tool) program (Altschul et al., 1990) and, for cytochrome oxidase 1, the Barcode of Life Data System (BOLD) program available online (https://www.boldsystems.org/). The phylogenetic trees were also generated with MEGA X, and the network diagram showing the relationships between haplotypes was generated with the PopART software (Bandelt et al., 1999; Clement et al., 2002) using median-joining network.

3. RESULTS AND DISCUSSION

3.1. Volga pikeperch

3.1.1 Microsatellite tests on volga pikeperch

In the case of the volga pikeperch, 13 microsatellite markers (described in the pikeperch species) (MS 420, MS 417, MS 192, MS 384, MS 373, MS 703, MS 701, MS 704, MS 404, MS 395, MSL-1) were selected for the studies. During the PCR optimization, I mainly varied the amount of $MgCl_2$, the application temperatures and the cycle numbers to create the ideal reaction conditions. In the case of two markers (MS 423, MS 424), repeated changes to the protocol failed to yield a product. Six markers (MS 420, MS 417, MS 192, MS 384, MS 373, MS 703) were found to be monomorphic or to have only two allele sizes at most by capillary gel electrophoresis. For the MS 420 marker these were 161 and 171 allele sizes, for MS 417 266, MS 192 223, MS 384 266, MS 373 180 and 190, and MS 703 only detected alleles of 170 bp. Finally, I performed microsatellite analysis using 4 markers (MS 701, MS 704, MS 404, MS 395) from the Kánainé's perch markers (Kánainé et al., 2019a) and the MSL-1 marker (Kohlmann & Kersten, 2008), described and adapted also for perch from the literature, demonstrating the adaptability of the markers between species.

In the 3 populations studied (Lake Balaton, Danube, Tisza and Holt-Körös), I was able to detect the most alleles (n=11) with markers MS 704 and MS 395. In 3 cases (MS 701, MS 704, MS 395), the allele sizes with the highest frequency per marker are above or close to 50%. When testing the applicability of a microsatellite marker, such a high allele frequency is not necessarily a good thing. If there are more allele sizes and a more diverse distribution, the polymorphism of the marker is also higher. Based on the global PIC (polymorphic information content) values, all the markers I used fell into the highly informative category. The PIC for MS 701, like MS 704, was 0.63. MS 404 was 0.76, MS 395 was 0.71 and MSL-1 was 0.77. When we look them separately by population, all three populations had markers that were only moderately informative in that population. These are MS 701 for Lake Balaton, MS 704 and MS404 for Tisza and Holt-Körös, and MS 404 and MS 395 for Danube.

In the case of the examined volga pikeperch, for all the individuals, it can be said that I did not observe a significant difference from the Hardy-Weinberg equilibrium in the case of any of the markers. Examining the 3 volga pikeperch populations separately, however, I found a deviation from the ideal condition in several cases. Only in the case of the low sample Tisza_Holt-Körös population occurred non-significant differences in the case of markers MS 701 and MS 395. Heterozygote deficiencies are observed in both Lake Balaton and the Danube. The Fis value measuring the differentiation within populations was 0.403 for the Balaton, 0.351 for the Tisza_Holt-Körös group and 0.447 for the Danube volga pikeperch samples. Based on the data, Tisza_Holt-Körös appeared to be the most diverse of the three

populations of volga pikeperch at the individual level. The coefficient took a positive value for all three populations, supporting the results of the test of significance for deviation from Hardy-Weinberg equilibrium and heterozygote deficiency. The Structure analysis for the 3 sampling sites (Lake Balaton, Tisza and Holt-Körös combined, Danube) considered the separation of 2 clusters the most likely out of 12 runs based on the Evanno method (Evanno et al., 2005).

3.1.2 Mitochondrial marker studies in the volga pikeperch

I examined the mitochondrial genomes of the three populations of volga pikeperch (Lake Balaton, Tisza-Holt-Körös, Danube) in the so-called control region, i.e. the D-loop region. The analysis was carried out on 68 samples from Lake Balaton, 10 from the Tisza_Holt-Körös unit and 34 from the Danube. PCRs were successful on all samples. After sequencing the alignment, I had 849 base pairs length sequences per sample available for analysis. Of the 849 bases tested, 8 bases were polymorphic and all 8 were found to be parsimony informative sites.

The haplotype diversity value (Hd) for the 3 populations together is 0.6049, while the nucleotide diversity value (π) is 0.001. When looking at each population separately, the nucleotide diversity of the Balaton population (π: 0.001 and Hd:0.65) are similar to those of the three populations together. For the Tisza and Holt-Körös π:0.0006, Hd:0.53, while for the Danube π:0.005 and Hd:0.43. In total, I was able to isolate 7 haplotypes. Individuals from Lake Balaton were found in all haplotypes. I found 3 haplotypes in the Danube population and 2 haplotypes in the Tisza-Holt-Körös unit. The most common haplotype 1 (Hap_1) was carried by 64 volga pikeperch individuals, including a mixture of samples from all three populations (Balaton-Bal, Tisza_Holt-Körös-THK, Duna-Du). Besides the Hap_1 haplotype, Hap_2 was the other haplotype present in all populations. This low level of mitochondrial diversification may be related to the high heterozygosity measured in previous microsatellite studies, where the measure of within-population differentiation (Fis) showed the lowest diversity for the Tisza-Holt-Körös unit.

The largest genetic distance (Fs: 0.001137) is observed between the Tisza_Holt-Körös and the Balaton population. The negative Fu Fs value for Balaton (-0.89) indicates an increasing population size, which is supported by the Tajima D value of -0.67. At the national level, FAO statistics show similar data, where the national catch increased from 11 tons to 13 tons over the sampling period (2016 and 2017). While this is an encouraging result, the quantity is still low compared to the 18 tonnes recorded in 2015 (FAO, 2019). Sequence checking in NCBI databases using nucleotide BLAST analysis confirmed the identification of individuals by morphological traits.

3.1. Crucian carp

3.2.1. Examination of the D-loop region of the crucian carp

In the case of the crucian carp, the mitochondrial D-loop, i.e. the control region, of 132 individuals from 6 sampling sites (Siófok (n=29), Ingó (n=18), Kányavár (n=17), Hőgyész (n=30), Siófok-Töreki lakes (n=19), Őszödi-berek (n=19)) was examined. From the 43 polymorphic base sites, I was able to identify 22 haplotypes out of 132 sequences. Out of the 22 sequence variants, 9 haplotypes are present in only one individual and 7 are present in only two samples, compared to the most common haplotype 1 (HapDl_1), which is present in 46

individuals. The third most abundant haplotype, haplotype 21 (HapDl_21), occurred only in samples from the Őszödi-berek. This population is the only one that is divided into two completely distinct haplotypes, HapDl_21 with 17 samples and HapDl_22 with 2 samples. The population sampled from Lake Balaton at Siófok carried the highest number of haplotypes, 9 in total.

The highest haplotype diversity value (Hd:0.83) was found in sampled individuals from Siófok, while for nucleotide diversity the Siófok-Töreki lakes crucian carp population showed the highest value, with π: 0.13. Although the Siófok-Töreki samples were only classified into 4 haplotypes, two individuals were classified into haplotype 4 (HapDl_4). This sequence variant contained the most polymorphic bases compared to the other haplotypes.

The nucleotide database comparison sequence analysis (BLAST-Basic Local Alignment Search Tool) available in NCBI GenBank, was used as a reference for the taxonomic classification. In standard nucleotide BLAST analysis of the crucian carp haplotypes, the sequences of 7 haplotypes (HapDl_3, HapDl_4, HapDl_6, HapDl_8, HapDl_11, HapDl_13, HapDl_14) out of 22 showed greater similarity with other members of the Carassius-complex, than with crucian carp. Based on the alignments, HapDl_3, HapDl_6, HapDl_8, HapDl_11, HapDl_13 and HapDl_14 all cover *Carassius auratus auratus*, the goldfish, and HapDl_4, *Carassius auratus buergeri*, which is a Japanese subspecies of goldfish. To confirm the taxonomic classification of our own haplotype sequences and to verify the function of the control region as a marker for taxonomic segregation, sequences carrying each D-loop haplotype were also tested for the mitochondrial genome Cytochrome *b* (Cyt.B.) and Cytochrome oxidase subunit 1 (Co.I.).

3.2.2. Investigation of the cytochrome *b* region of the crucian carp

In the cytochrome *b* analyses, the DnaSP6 software identified only 6 haplotypes out of the 22 haplotype reference individuals (determined from the previous control region) based on nucleotide diversity. The D-loop haplotypes HapDl_4, HapDl_6 and HapDl_11, which were identified by the previous BLAST analysis with the *Carassius auratus auratus* and *Carassius auratus buergeri* sequences, were also separated here based on their nucleotide diversity in the cytochrome *b* region of the mitochondrial genome. The also different haplotypes HapDl_8, HapDl_3 and HapDl_14 were included in the most abundant cytochrome *b* group HapCb_1. BLAST analysis of the cytochrome *b* groups showed a much more consistent picture. All sequences were aligned with *Carassius gibelio* sequences, in addition, in contrast to the D-loop value, in most cases with 100% agreement for both the query and identity values.

3.2.3. Examination of the crucian carp cytochrome oxidase 1.

Of the 3 regions of the mitochondrial genome, the Co.I. marker showed the fewest haplotype groups, with only 4 haplotypes. The BLAST analysis of the 4 Co.I. groups highlights the limitations of online databases and the criticality of the uploaded data. NCBI BLAST standard nucleotide analysis also identified 2 haplotypes (Co.I._1, Co.I._3) as *Carassius auratus.* The cytochrome oxidase 1 region is also referred to as the barcode region. A separate database has also been created for searching by sequence. In BOLD (The Barcode of Life Data System) (web) it is also possible to search for identities based on the FASTA format alignment of sequences. The results overlapped in the two databases, while Co.I._1

and Co.I._3 were identified, as *Carassius auratus*, Co.I._2 and Co.I.-4 were identified as *Carassius gibelio*. Sequence identifications should be checked with great care and with as many molecular genetic markers as possible, because informatics databases are not yet able to independently detect errors, resulting from inaccurate uploads, poorly defined sequence sequences and analysis of samples from individuals with incorrect taxonomic units.

3.3 Sichel

3.3.1 Adaptation of microsatellite markers to the species sichel

As the sichel (Pelecus cultratus) has not been studied with microsatellite markers yet, very few preliminary studies or baselines were available about the species. In addition to mitochondrial markers, my aim was a more comprehensive species-specific study with microsatellite markers. Since interspecific marker adaptation has been shown to work in the volga pikeperch, I first tried to adapt already working microsatellite markers from closely related species (Baerwald & May, 2004; Barinova et al., 2004; Urbánková et al., 2013; Hosseinnia et al., 2014). As in the case of the volga pikeperch, the variable values used to optimize the PCR protocol were mainly the amount of $MgCl_2$, the primer annealing temperature and the number of cycles.

A total of 11 microsatellite markers from the literature were tested. Out of the 11 markers, PCR products were successfully amplified for 5 markers (Albi22, Albi61, Albi462, Gob28, CypG24) after optimization of the protocols, but for 2 of these markers (Albi462, CypG24), so many other fragments were accumulated that the results were unreliable and further optimization failed to reduce the amount of "noise". Three markers (Gob28, Bl1, Albi22) were monomorphic based on capillary gel-electrophoresis results. Only one microsatellite marker, Albi 61, was found to be polymorphic, but it had only 3 alleles. A single three-allele microsatellite marker alone is not suitable for population research.

3.3.2. Mitochondrial examination of the sichel

Examining the mitochondrial D-loop region of the three populations (Lake Balaton, Fertő, Poland) of sichel (*Pelecus cultratus*), I clarified and aligned 125 sequences. Software analysis of the resulting 803 bp long consensus sequences revealed 5 haplotypes. Of the 4 polymorphic base sites defining the 5 haplotype groups, just two were phylogenetically informative positions. Based on both the sample numbers and the geographical distance between the sampling sites, this variability is extremely low, considering only the 22 haplotypes obtained with more than 8 samples of crucian carp. In addition, the majority of individuals (104 samples) carry the most common haplotype 1 (Hap_1) out of 125.

Samples from the Vistula-lagoon in Poland all belong to this most common group. The second haplotype (Hap_2) was obtained only from samples from Fertő, and the haplotypes Hap_3, Hap_4 and Hap_5 were carried only by Lake Balaton samples. Thus, the Balaton sichels have the highest number of haplotypes. Except for Hap_2, which is only found in the Fertő samples, all haplotypes contain a Balaton sample, including the two rare Hap_4 and Hap_5. In the case of the completely homogeneous population from the Polish Vistula-lagoon, calculations of genetic differentiation within the population are meaningless, as no variation between the individuals was detected based on the sequences examined.

The value of haplotype diversity (Hd) is 0.7 (± 0.05) for Fertő and 0.41 (± 0.08) for Balaton. Although the samples from Fertő only carry two haplotypes, they are half the number (n:23) of the samples from Balaton. The Fu' Fs value shows the largest differences between the two populations. While in the Balaton population it was -4.09, in the Fertő population it was positive (1.28). The negative value for Lake Balaton could be an indication of a recent increase in the population. Based on Fu's Fs, it can be assumed that the population in the case of the Fertő has undergone a bottle-neck effect before the 2017 sampling year. Since the Fertő population of sichel is a native, self-sustaining population, a possible disease or any external factor that causes a decline in numbers could easily reach this effect. Tajima's D-value recorded a value of -1.67 for Balaton and a positive value of 1.23 for the Fertő population. The negative value in population dynamics studies may be due to an increase in mutation rate, which is consistent with the increasing population, estimated from the positive Fu' Fs value.

4. CONCLUSIONS AND SUGGESTIONS

4.1. Marker adaptability between related species

In the case of the volga pikeperch (*Sander volgensis*), marker adaptation was successful overall. Of the 13 pikeperch microsatellites selected, only 2 markers (MS423, MS424) were found to amplify product during PCR, even after repeated changes in protocols. Six markers (MS 420, MS 417, MS 192, MS 384, MS 373, MS 703) that were found to be polymorphic in the pikeperch (Kánainé et al., 2019a), were found to be monomorphic or carried only 2 alleles in the volga pikeperch species and thus not applicable for measuring population diversity in this species. Based on the PIC values, the 5 sufficiently polymorphic markers (MS 701, MS 704, MS 404, MS 395, MSl-1) were all found to be suitable for the study of Hungarian volga pikeperch populations. However, the results suggest that not only the degree of polymorphism but also the allele sizes detected by each microsatellite marker may differ during marker adaptation between different species.

For more comprehensive studies on the genetic background of the volga pikeperch populations, the inclusion of additional nuclear markers is recommended, including the adaptation of additionally available pikeperch markers.

Marker adaptation has been more difficult in the case of the sichel (*Pelecus cultratus*). It doesn't have any as close relative as the pikeperch to the volga pikeperch, because the sichel is the only member of the genus *Pelecus*. Based on literature data, 11 markers were selected from 5 closely related species of the carp family, namely the schnedier (*Alburnoides bipunctatus*), the common sturgeon (*Rutilus rutilus*), the roach (*Cyprinus carpio*), the gudgeon (*Gobio gobio*) and the *Leuciscus souffia*.

One marker alone is not enough to characterise a population, but in the future, if we can adapt new markers or isolate them by library construction, we will be able to do a more comprehensive study. This work demonstrates that marker adaptation is not impossible, even for species that belong to a separate genus. Although most of the markers, for that a working PCR protocol has been established in this species, have been found to be monomorphic in the tested stocks, they may be polymorphic in other, more genetically diverse stocks.

4.2. Population genetics studies of the volga pikeperch

The 5 polymorphic microsatellite markers (MS 701, MS 704, MS 404, MS 395, MSL-1) and the mitochondrial genomic control region (D-loop) isolated from perch were found to be suitable for genetic diversity analysis of samples from the populations of Lake Balaton, Danube and the pooled Tisza and Holt-Körös samples. In the case of microsatellites, it would be usefull to include more markers in subsequent studies and to collect more samples from the Tisza and Holt-Körös areas, but this is more difficult to do for such a large area. Since the volga pikeperch is very popular among anglers, it would be worthwhile to contact the angling associations in the area in the future.

Considering both the number of unique alleles detected for microsatellite markers and the haplotypes determined from the D-loop region of the mitochondrial genome, it can be concluded that the Balaton volga pikeperch population contains individuals with rare genetic backgrounds, that make it a valuable genetic resource for the recruitment of small populations with potentially limited genetic backgrounds or for the establishment of new populations.

The genetic distances between populations are smaller than expected but proportional to the geographical distances. The two geographically most distant populations have the largest genetic difference. This may be the result of stocking for angling purposes using few breeding animals, possibly of Danube or Balaton origin.

Since we already have working markers and protocols, it would be worthwhile to conduct a second round of sampling and testing, including more individuals and populations, based on the results. This would answer the question of the impact of the uncontrolled stockings to date on the natural populations of the volga pikeperch, or whether the diversification of the populations at the time of sampling has continued to decrease, and as the last catch data in the FAO data is currently 2018, it would be necessary to update these data to provide a more comprehensive view of the status of the domestic stocks.

4.3 Population genetic examination of crucian carp

Of the 22 haplotypes obtained in the mitochondrial analysis, more than half were present in only 1 or 2 samples, indicating a diverse origin of the native population. The results obtained show the complete isolation of the population of the Őszödi-berek. The fish sampled here carry only two haplotypes that are completely distinct from the rest of the population. Őszödi-berek is now a completely isolated population, not only geographically but also genetically. The reason why the Hőgyész, which is also unrelated to other sampling sites, carries a mixture of haplotypes, and why a crucian carp population which is unique to that lake has not developed there is not known, but can most probably be explained by the stocking in the lake and its water system.

The individuals with the most divergent sequence from the others, based on nucleotide diversity, were in the Siófok-Töreki population. This population showed that the taxonomic questions in the literature about the members of the *Carassius*-complex have validity in our country too. This is indicated by the results of the BLAST analysis, which revealed the presence of 5 *Carassius auratus auratus* haplotypes with phenotypically distinct crucian carp samples, as well as the *Carassius auratus buergeri* haplotype. A BLAST search based on the cytochrome *b* marker identified all previous D-loop haplotypes as *Carassius gibelio*, according to the phenotype.

Cytochrome oxidase 1 region alignment in the BOLD system and BLAST analyses also indicated the goldfish origin of some of the haplotypes, as did the D-loop. This could be a consequence of hybridization between goldfish and crucian carp, a phenomenon that is well known and now already proven in our country. In the case of individuals as these, it would be recommended that nuclear DNA be also tested. However, it is also possible that incorrectly recorded or identified sequence data may have been included in the databases, leading to incorrect taxonomic identification. Based on these results, the use of multiple markers and databases is recommended for the identification of sequences of dubious origin in crucian carp studies.

Based on the present results, it is very likely that hybridisation between the members of the *Carassius*-complex has taken place in the studied water systems, and that this is responsible for the appearance of the *Carassius auratus aruatus* and *Carassius auratus buergeri* sequences in the samples.

4.4. Population genetics studies of sichel

Results of examination the mitochondrial genome control region in the three sichel populations are concerning. The native populations of the sichel, considered to be a species of community importance, showed extremely low diversity according to the mitochondrial D-loop marker. The only 5 haplotypes identified from the sequences of 125 individuals are very few. More than 85% of the samples carry the same sequence variation, which is very surprising considering that some of the samples come from the reference population of Vistula-lagoon in Poland, which is geographically quite distant from the other populations. Nevertheless, the Polish samples were not separated from the Hungarian ones, but were grouped into a single haplotype, the most common one. The population in Ferto shows higher diversity at this level, where individuals are grouped into two distinct haplotype groups. Although the two haplotypes are also not very advantageous from a diversity point of view (suggesting that only two ancestral maternal lines are present in the whole population studied), especially in an isolated population. It should be pointed out that one of these groups (Hap_2) is unique to Fertő and therefore very valuable.

In the future, it would be worthwhile to adapt more microsatellite markers and to collect more information on the populations of the garda in the study areas in a larger scale work. To develop new SNP or microsatellite markers, and also to study the nuclear genome of the sichel population with them. If such low diversity is also observed for nuclear markers, it would be worthwhile to take gene conservation steps for the protection of the species. The conservation of the species is a priority for the European Community, as it is a species of Community importance. If the genetic homogeneity of the stock is further confirmed, it means that the emergence of even a single unknown disease could threaten the whole stock.

5. NEW SCIENTIFIC RESULTS

New scientific findings from my research:

1. By optimizing PCR protocols, I successfully adapted 11 microsatellite markers (MS 420, MS 417, MS 192, MS 384, MS 373, MS 703, MS 701, MS 704, MS 404, MS 395, MSL-1) from pikeperch (*Sander lucioperca*) to volga pikeperch (*Sander volgensis*) and 1 microsatellite marker (Albi61) from schnedier (*Alburnoides bipunctatus*) to sichel (*Pelecus cultratus*).

2. Population genetics of 3 Hungarian volga pikeperch stocks (Balaton, Danube, Tisza_Holt-Körös) were examined using five newly adapted and polymorphic microsatellite markers (MS 701, MS 704, MS 404, MS 395, MSL-1) and the mitochondrial genome control region. I found that the populations deviate from the Hardy-Weinberg equilibrium and that the Balaton population carries the most unique, and thus the most valuable genetic background from a gene conservation point of view.

3. Using three mitochondrial genetic markers (cytochrome *b*, cytochrome oxidase subunit 1, D-loop), which have been well characterized in the literature, I performed phylogenetic analysis of 5 and one additional crucian carp (*Carassius gibelio*) populations from Lake Balaton and its catchment. I determined the marker (Cyt.B) most suitable for the taxonomic identification of the members of the *Carassius*-complex.

4. I have proved that individuals carrying mitochondrial genomes of goldfish origin can be found in the Hungarian silver carp population.

5. I was the first in the world who made a population genetics study on 2 Hungarian and 1 Polish population of the sichel. I found that both the Hungarian and the Polish populations have a worryingly homogeneous genetic background.

6. THE AUTHOR'S PUBLICATIONS RELATED TO THE SUBJECT OF THE THESIS

International journal

Ősz Á., Horváth Á., Hoitsy Gy., Kánainé Sipos D., **Keszte Sz.**, Sáfrány J., Maric S., Palkó Cs., Tóth B., Urbányi B. (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 Paper: e5152, 26 p. (2018)

Keszte Sz., Ferincz Á., Tóth-Ihász K., Balogh R., E., Staszny Á., Hegyi Á., Takács P., Urbányi B., Kovács B. (2021): Mitochondrial sequence diversity reveals the hybrid origin of invasive gibel carp (*Carassius gibelio*) populations in Hungary. PeerJ, 9, e12441. https://doi.org/10.7717/peerj.12441

National journal

Keszte Sz., Stein R., Kánainé S. D., Balogh E., Zellei Á., Sebestyén A., Balogh R., Guti Cs. F., Bokor Z., Urbányi B., Kovács B. (2017): Mitokondriális genetikai vizsgálat a velencei-tavi vadponty anyajelöltjeinek állományán; Animal welfare, Etológia és Tartástechnológia; 2017. XIII/2 p. 74-80

Keszte Sz., Mészáros O., Kánainé S. D., Balogh E., Zellei Á., Sebestyén A., Balogh R., Guti Cs. F., Bokor Z., Urbányi B., Kovács B. (2017): Velencei ponty állomány diverzitásának mikroszatellit markerekre alapozott genetikai vizsgálata; Animal welfare, Etológia és Tartástechnológia; 2017. XIII/2 p. 68-73

Keszte, Sz.; Kánainé, S. D.; Stein, R.; Mészáros, O.; Balogh, E.; Zellei, Á.; Sebestyén, A.; Balogh, R.; Guti, Cs F.; Bokor, Z et al. (2018): Velencei-tavi vadponty tájfajta Kajászói Tógazdaságban fenntartott anyajelölt állományának genetikai diverzitás vizsgálata HALÁSZAT 4.: 1 pp. 3-9., 7 p.

Conference publication

Foreign conference publication (oral presentation):

Keszte Sz., Tóth-Ihász K., Balogh R., Ferincz Á., Staszny Á., Józsa V., Urbányi B., Kovács B. (2019): Molecular genetic diversity of hungarian silver prussian carp carassius auratus gibelio populations based on mitochondrial d-loop sequences. (oral presentation), Aquaculture Europe 2019, Internration Conference and Exposition, Berlin, Germany, 2019 October 7-10., Book of abstracts pp. 677-678

National conference publication (presentation):

Keszte Sz.: Süllő (*Sander lucioperca*) mikroszatellit markerek alkalmazásának lehetősége a kősüllő (*Sander volgensis*) genetikai diverzitás vizsgálatára; Tavaszi Szél Konferencia (Doktoranduszok Országos Szövetsége); Győr, 2018. május 4-6; Book of abstracts pp. 50

Keszte Sz, Balogh R, Dr. Bernáth G, Dr. Bokor Z, Molnár J, Várkonyi L, Dr. Ferincz Á, Dr. Staszny Á, Dr Józsa V, Dr. Urbányi B, Dr. Kovács B: Molekuláris módszerek fejlesztése a Balaton közösségi jelentőségű halfajainak populáció genetikai vizsgálatához; XXXVII. Óvári Tudományos Napok; Mosonmagyaróvár; 2018. november 9.-10. Book of abstracts pp. 150-150., 1 p.

Keszte Sz, Tóth-Ihász K., Balogh R. E., Dr. Ferincz Á., Dr. Stasznyy Á., Dr. Urbányi B., Dr. Kovács B.: A balaton ezüstkárász (*Carassius auratus gibelio*) állományának genetikai diverzitás vizsgálata mitokondriális genomi vizsgálatok alapján, XXII. Tavaszi Szél Konferencia, Debrecen; 2019. május 3-5; Book of abstracts pp 62.

Keszte Sz, Tóth-Ihász Katalin, Balogh Réka Enikő, Ferincz Árpád, Stasznyy Ádám, Józsa Vilmos, Urbányi Béla, Kovács Balázs: Magyarországi ezüstkárász (*Carassius auratus gibelio*) populációk genetikai diverzitás vizsgálata, (VII. Gödöllői Állattenyésztési Tudományos Nap, 2019. november. 22; Book of abstracts p. 52

National conference publication (posters):

Keszte Sz., Balogh E., Ősz Á., Uri Cs., Guti Cs., Bokor Z., ifj. Radóczi J., Urbányi B., Kovács B.: Sügér állományok diverzitásának mitokondriális DNS-re alapozott molekuláris genetikai vizsgálata; XXXVI. Óvári tudományos nap. Mosonmagyaróvár; 2016. november 10. Book of abstracts pp. 286-287., 2 p.

Keszte Sz, Kánainé S D, Balogh R, Bokor Z, Bernáth G, Várkonyi L, Urbányi B, Kovács B.: Multiplex PCR módszer fejlesztés kősüllő (*sander volgensis*) genetikai diverzitás vizsgálatához; XLII. Halászati Tudományos Tanácskozás; Szarvas, 2018. május 30-31, Book of abstracts p. 52

Keszte Sz., Tóth-Ihász K., Balogh R., Dr. Ferincz Á., Dr. Stasznyi Á., Dr. Bokor Z., Dr. Bernáth G., Várkonyi L., Dr. Józsa V., Dr. Urbányi B., Dr. Kovács B.: A balatoni ezüstkárász (carassius auratus gibelio) populációk genetikai diverzitás vizsgálata a mitokondriális genom szekvenciák alapján; XLIII. Halászati Tudományos Tanácskozás; Szarvas, 2019. május 29-30. Book of abstracts p. 99-100.