Doctoral (PhD) dissertation

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Population genetic structure and diversity of endangered Hungarian Tench (*Tinca tinca* L.1758) and Crucian carp (*Carassius carassius* L.1758) populations, with reference to the taxonomic status of two *Carassius* species—a foundation for conservation strategies

By

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1. BACKGROUND AND OBJECTIVES

Crucian carp (*Carassius carassius* L. 1758) and tench (*Tinca tinca* L. 1758) are cyprinid fish native European waters (Brylinska et al., 1999; Copp et al., 2008). They are highly adaptable to a wide range of temperatures and low oxygen content in the water in both summer and winter. This ability to survive in anoxic conditions and at high densities in small bodies of water (Sollid, 2005; Kottelat and Freyhof, 2007) may allow tench and crucian carp to outcompete native species that are more sensitive to oxygen depletion and hence unable to thrive in such habitats when moved outside their native ranges. These characteristics make these species ideal candidates for adaptability to a wide range of environmental conditions. Because of their many advantages for aquaculture and also because of their taste and popularity among sport anglers (Szczerbowski and Szczerbowski, 2002; Ćirković et al., 2012), their production has increased in Europe. Nowadays, the entire economic value and contribution of these species to the fisheries sector are still limited. Nevertheless, climatic change may make these species ideal for future Hungarian aquaculture production. The numbers of these fishes are declining in Europe, and the main reasons for their decline are habitat loss and anthropogenic factors (Copp, 1991; Sayer et al., 2011; Simic et al., 2013). However, hybridization with closely related fish species that are not native, has also contributed to the decline in the number of crucian carp populations (Copp & Sayer, 2020). Well-documented data has shown that gibel carp (*Carassius gibelio* Bloch, 1782) competes with native fish and threatens the reproductive capacity of native crucian carp, causing species displacement (Smartt, 2007; Copp et al., 2010; Mezhzherin et al., 2012; Wouters et al., 2012). Furthermore, the identification of pure-bred *Carassius carassius* and hybrids using external morphological investigation is challenging, making these threats a concern throughout Europe (Hanfling et al., 2003). This significant morphological similarity between these species has hampered important insights into their taxonomic, biogeographic, and introduction histories (Rylková et al., 2013). However, despite the fact that these non-native species may cause the decline of native species or even their extinction (Pyšek et al., 2017; Dueñas et al., 2018), species invasion gives us the opportunity to consider the many different evolutionary processes that occur within species (Fitzpatrick et al., 2010).

According to Faulks et al. (2017) understanding the diversity, genetic structure, and high-quality habitat selection of endangered species is essential for participating in successful conservation and management programmes. Species' genetic diversity serves as a springboard for systematic action

planning aimed at preserving the species and reducing the risk of extinction (Souza-Shibatta et al., 2018). The structure of the population is the result of both current and historical processes. Therefore, in order to better understand how population diversity and species subdivision are constructed, it is also important to know their history. Pleistocene glacial cycles, in particular range shifts during the recolonization of glacial refugia in Europe, influenced the distributions and genetic diversity of present European species (HEWITT, 1999). Like most freshwater fish species in Eurasia, crucian carp and tench exhibit distinct phylogeographic subdivisions within their geographic ranges (Hewitt, 2004). Recent phylogeographic studies have found that the tench is separated into highly divergent Western and Eastern geographical clades based on analysis of nuclear and mitochondrial DNA sequence markers (Lajbner & Kotlík, 2011; Lujić et al., 2017; Karaiskou et al., 2020). On the other hand, human-aided transmissions may lead to introgressions across phylogroups, resulting in phylogeographic patterns that do not reflect natural historical processes (Lajbner et al., 2011). Likewise, there are two distinct European lineages of *Carassius carassius*, one found throughout the Northern, Central-Eastern drainages, the other nearly entirely limited to the Danubian catchment (Jeffries et al., 2016).

The availability of genome wide molecular markers, either protein or DNA (mitochondrial DNA or nuclear DNA), has already aided the identification of fish species, resolving taxonomic ambiguity and phylogenetic relationships and providing a deeper knowledge on their diversity, population, and genetic structure (Liu & Cordes, 2004; Tanya & Kumar, 2010; Hakim & Ahmad, 2017). Despite the fact that few studies on the population structure, genetic diversity, and phylogenetic relationships among populations of these species have been conducted, polymorphism of microsatellite markers (Kohlmann et al., 2010; Presti et al., 2010; Janson et al., 2015; Al Fatle et al., 2022), mitochondrial DNA (mtDNA) (Apalikova et al., 2011; Lajbner and Kotlík, 2011; Lo Presti et al., 2014; Knytl et al., 2018; Al Fatle et al., 2022), and a set of SNPs (Jeffries et al., 2016; Kumar et al., 2019) has been shown to be an excellent tool for detecting population structure, phylogeny, and biogeographic history in the above two species populations.

In the present study, a combination of microsatellite DNA markers, mtDNA Cytb gene sequences, mtDNA Cytochrome C Oxidase I (COI) gene sequences, and two nuclear markers (*Act*) and (*RpS7*) were used to meet the following objectives:

- To estimate the population structure and genetic diversity of seven wild populations of tench in Hungary, as well as eight wild populations and three stocks of crucian carp. Furthermore, we aimed to investigate the genetic differentiation between wild and cultured populations of crucian carp. Thus, providing essential knowledge for the development of successful selective breeding strategies on the one hand and sustainable conservation strategies on the other. In the case of crucian carp, the extent of hybridization with gibel carp, *Carassius gibelio* (Bloch, 1782), is also the focus of our research.
- To provide a systematic phylogeography of the wild tench populations as the basis for management and conservation efforts and for the purpose of determining population origins for potential translocations.
- To investigate the genetic divergence and phylogenetic relationship between *Carassius carassius* and the closely related *Carassius auratus gibelio,* and to estimate their taxonomic status in the genus *Carassius* using nucleotide sequence difference data from the mtDNA Cytochrome C Oxidase I (COI) region. Understanding the taxonomic status of these species in Hungarian waters can benefit conservation efforts.
- To achieve the stated goals, ex-situ genetically described live GenBank of the two species must be established and developed. Identified hybrids should be excluded from GenBank activities.

2. MATERIALS AND METHODS

2.1 Target fish species

This study focused on two cyprinid fish species: crucian carp (*Carassius carassius* L. 1758) and tench (*Tinca tinca* L. 1758).

2.2 Sample collection, and DNA extraction

320 crucian carp fin tissue samples were collected from eleven different fish stocks in Hungary, representing eight wild populations and three farmed stocks, as well as 175 tench samples from seven different sites in the country's eastern, central, and western regions. Figure 1 and 2 depict the sample sites.

The morphological identification of the species was carried out by experts using simple external investigation. The fish were handled carefully and, after sampling, were released into their natural habitat. Fish were anesthetized with clove oil prior to sampling. The fresh fin tissue samples were kept in 2 ml Eppendorf tubes containing 70% ethanol, then subsequently labelled and transported to the molecular laboratory located at the National Centre for Biodiversity and Gene Conservation (NBGK-HGI). At the institute, these Eppendorf tubes were kept in a conventional freezer at a temperature of -20 C. The whole genomic DNA was extracted from the fin clips using a modified salting out protocol based on Miller et al. (1988). The procedure was modified in the NBGK-HGI molecular genetic laboratory. The protocol was carried out over the course of three days. A spectrophotometer (NanoDrop 2000c, Thermo- Scientific, Waltham, MA, USA) was used to measure the concentration of the extracted DNA, and then the quality was checked on a 2% agarose gel.

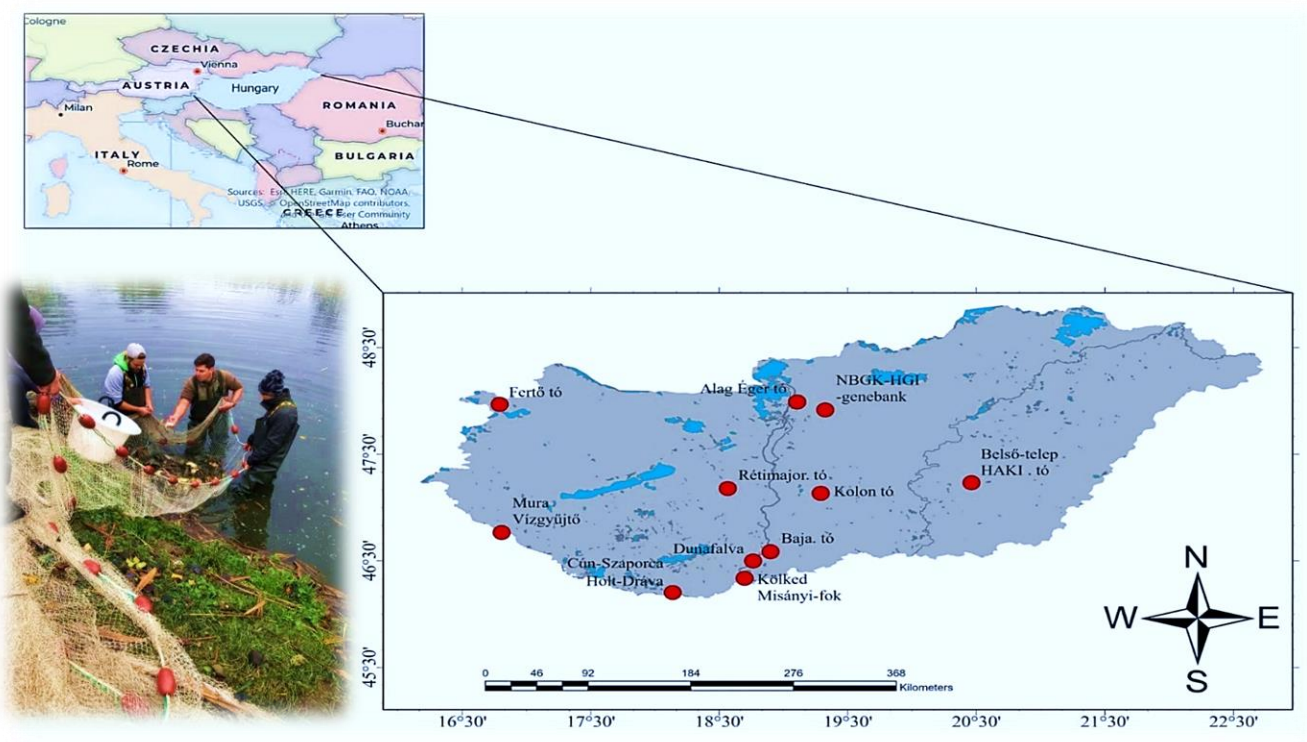


Figure 1. Crucian carp sample locations map.

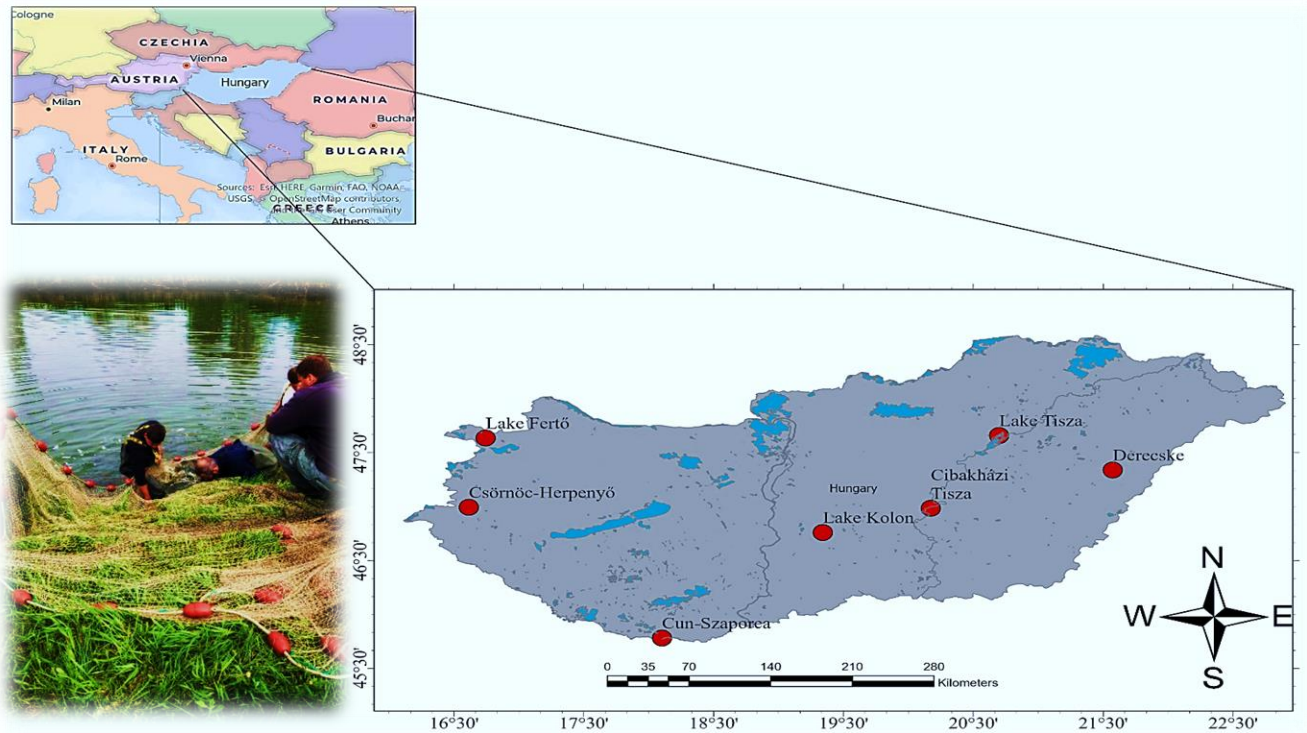


Figure 2. Tench sample locations map.

2.3 Molecular genetic methods and data analysis

2.3.1 Microsatellites analysis

A total of twelve within-and cross-species microsatellite markers were used to genotype all 175 tench samples. Seven markers, MTT1-3, MTT5-6, and MTT8-9, were developed for tench (Kohlmann and Kersten, 2006); two markers, MFW1 (Crooijmans et al., 1997) and CypG24 (Baerwald and May, 2004) were developed for common carp; and three new markers were developed for tench in this study (MT1-3). These markers were developed by running Primer 3 software on sequences of the tench transcriptome that have repeat motifs. In the case of crucian carp, 13 microsatellite markers (MFW7, GF1, GF29, YJ0010, YJ0022, HLJYJ017, HLJYJ028, HLJYJ029, HLJYJ041, HLJYJ046, HLJYJ082, J62, and CypG24) originally developed for other cyprinid species were amplified, as described by Zheng et al. (1995), Crooijmans et al. (1997), Yue & Orban (2002), Baerwald & May (2004), Guo & Gui (2008), and Zheng et al. (2010).

All PCRs were conducted according to published protocols. However, before the actual amplification, the protocol for each marker was changed and done more than once. Amplified fragments were analyzed using an ABI Prism 3130 Automated Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). As an internal standard, an ABI 3130 automatic fragment analyzer used a Pop 7 polymer, a 50 cm capillary array, and a GS500-LIZ molecular size standard.

2.3.2 Restriction fragment length polymorphism (RFLP) analysis in Tench

The RFLP analysis was carried out on a total of 175 tench individuals utilizing two nuclear-encoded Exon-Primed Intron-Crossing (EPIC) makers: the second intron of the actin gene (*ACT*) and the first intron of the gene encoding the S7 ribosomal protein (*RpS7*). The EPIC primers were chosen to amplify a 335 bp nuclear DNA sequence encoding the second intron of the actin gene (*Act*) as designed by Touriya et al. (2003). Similarly, the EPIC primers described by (Chow and Hazama, 1998) were used to amplify another part of nuclear DNA, a 923–927-bp amplicon containing the ribosomal protein S7 (*RpS7*) second intron (Lajbner and Kotlík, 2011). Each PCR reaction was conducted in a 15 µl PCR mixture containing 30 ng/µl of genomic DNA, 10.7 µl DW, 1.2 µl 10X DreamTaq Buffer with 20 mM $MgCl_2$, 1µl (10 mM) dNTP mix, 0.5 µl of each specific primer (5 µM), and 0.1 µl Taq DNA polymerase (5 U/ µl) (Thermo Scientific, Waltham, NJ, USA). Chow and Hazama (1998) and Touriya et al. (2003) explain how two different PCR programs were used to multiply the products. Two different restriction enzymes were employed to digest the

amplified PCR products, which were selected based on previous findings (Lajbner and Kotlík, 2011). The *Act* gene was digested by Eco521 endonuclease, while the *RpS7* gene was broken down by NdeI endonuclease. At 37°C for 10 hours, 5 µl of the PCR products were digested in 11 µl volumes containing 9 µl of nuclease-free water, 1 µl of PCR buffer, and 1µl (10u/µl) of restriction enzyme Eco52I or NdeI (Thermo Scientific, Waltham, NJ, USA). They were then inactivated by incubation for 20 min at 65 °C. The products digested by the aforementioned enzymes were useful in predicting the haplotype patterns between western and eastern haplotypes based on the known DNA sequences of the amplified PCR products (Lajbner et al., 2010). Restriction fragments were separated electrophoretically on a 2% agarose gel containing GelGreen Nucleic Acid Stain (Biotium Inc, Landing Parkway Fremont, CA, USA). Subsequently, using VisionWorksLS analysis software (LTF Labortechnik GmbH & Co. KG., Wasserburg, Germany), the DNA fragment patterns were verified and photographed under UV light by a gel documentation system (Analytik Jena).

2.3.3 Mitochondrial DNA amplification and sequencing

2.3.3.1 Amplification and sequencing of the Mitochondrial Cytb gene in Tench

The universal primer pairs Glu-F (5′-AACCACCGTTGTATTCAACTACAA-3′) and Thr-R (5′ ACCTCCGATCTTCG-GATTACAAGACCG-3′) were used to amplify 175 tench Cytb (615-bp) gene sequences, which were designed by using the flanking tRNA sequences according to (Machordom and Doadrio, 2001).

The PCR reaction of each sample was carried out in a 15 µl PCR mixture containing 30 ng/µl of genomic DNA, 10.7 µl DW, 1.2 µl 10X DreamTaq Buffer with 20 mM $MgCl_2$, 1 µl (10 mM) dNTP mix, 0.5µl of each specific primer (5 µM), and 0.1µl (5 U/ µl) Taq DNA polymerase (Thermo Fisher Scientific, Waltham, NJ, USA). The following protocol was used to conduct PCR reactions in the Kyratec PCR thermal cycler (Applied Biosystems, Foster City, CA, USA): Initial denaturing at 95°C for 5 min, then 2 cycles at 94°C for 1 min, annealing temperature at 60°C for 1 min 30 s, 72°C for 2 min, then another 30 cycles at 94°C for 1 min, 1 min 30 s at 54°C, and 2 min at 72°C, then final extension for 10 min at 72°C. Successful PCR products were purified by NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel, Düren, Germany) before being sequenced. On a 2% agarose gel conducted in TBE buffer, the quality of the purified products was assessed. The purified products were then sequenced using an automated genetic analyzer, ABI 3130 (Applied

Biosystems, Foster City, CA, USA), with a POP7 polymer and a 50 cm long capillary array, according to the manufacturer's instructions for the Big Dye terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

2.3.3.2 Amplification and sequencing of the Mitochondrial COI region in Crucian carp

For a total of 104 out of 320 crucian carp sampled from six wild populations and three farmed stocks in Hungary's eastern, central, and western regions, the Cytochrome Oxidase C subunit I gene (COI) was amplified for sequencing analysis using universal primer pairs CO1-FF2d-F (5′-TTCTCCACCAACCACAARGAYATYGG-3′) and CO1-FR1d-R (5′-CACCTCAGGGTGTCCGAARAAYCARAA -3′) (Ivanova et al., 2007). The amplification process was as follows: 94°C for 2 min, 30 cycles at 94°C for 40 s, 52°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 7 min. 15 µl of PCR mixture contained 30 ng/µl of genomic DNA, 10.9 µl of DW, 1.2 µl 10X DreamTaq Buffer with 20 mM $MgCl_2$, 1 µl dNTP (10 mM), 0.4 µl of each primer (10 µM), and 0.1 µl (5 U/ µl) of Taq DNA polymerase (Thermo Fisher Scientific, Waltham, NJ, USA). NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel, Düren, Germany) were used to purify the PCR products. Purified products were quality tested on a 2% agarose gel, and then sequenced with the Big Dye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The ABI 3130 genetic analyzer (Applied Biosystems, Foster City, CA, USA) was used to reveal the sequences.

2.3.4 Statistical analysis

The Genotyper 4.0 software package from Applied Biosystems was used to estimate fragment length. The parameters of genetic variance, including observed heterozygosity (Ho), expected heterozygosity (He), number of alleles (Na), and effective number of alleles (Neff), genetic variance (F_{st}), and heterozygote deficit, were calculated using GenAlEx6.5 (The Australian National University, Canberra, Australia) (Peakall and Smouse, 2006). MICRO-CHECKER version 2.2.3 (The University of Hull, Hull, UK) (number of randomizations: 1000, 95% CI) was used to detect possible genotyping errors, allele dropout and non-amplified alleles (Oosterhout et al., 2004). GENEPOP software (Raymond and Rousset, 1995; Rousset, 2008) was used to assess deviations from the Hardy–Weinberg equilibrium (HWE) for each locus in each population using a Markov chain (5000 dememorizations, 500 batches, 5000 iterations per batch) (Guo and Thompson, 1992). Genetic divergence between populations was assessed by estimating the pairwise F_{st} of Weir (1996) (Weir, 1996) as well as Cavalli-Sforza and Edwards (1967) (Cavalli-

Sforza and Edwards, 1967) genetic distance using FreeNA software (INRA, Montpellier, France) (Chapuis and Estoup, 2007). The ENA correction was used for the F_{st}, while the INA correction was used for genetic distance. For the computation of the bootstrap 95 % confidence intervals, 10,000 replicates were used.

The genetic relationship between populations and individual assignments of fish was inferred via a Bayesian clustering analysis using the statistical program STRUCTURE v2.3.3 (University of Chicago, Chicago, IL, USA) (Pritchard et al., 2000; Falush et al., 2003; Pritcharda et al., 2010). The admixture model was used to assume the structure analysis parameters, and the analysis was run 10 times per K with a burn-in of 10^4, followed by 10^5 Markov chain-Monte Carlo (MCMC) repetitions. STRUCTURE HARVESTER software (University of California, Santa Cruz, CA, USA) (Earl and vonHoldt, 2012) was used to calculate the most likely cluster number K, the posterior probability (highest LnP (D)), and the ΔK (Evanno et al., 2005).

Different software packages were used to analyze the mtDNA sequencing data. The haplotype diversity (Hd), nucleotide diversity (π), the number of segregating sites, and the total number of mutations for all populations and regions were estimated by DnaSP 5.10 software (Librado and Rozas, 2009). Using Mega-X 10.1 software (The Pennsylvania State University, University Park, TX, USA), DNA sequences were edited and aligned. NETWORK 10.0 software (Fluxus Technology Ltd., Colchester, England) (Bandelt et al., 1999) was used to generate haplotype network analyses for all populations in this study as well as GenBank sequencing. The phylogenetic trees were made using MegaX-11 software with Neighbour Joining fitting using the Bootstrap method with 1,000 bootstrap replicates, Tamura 3 parameter model Gamma-Distributed (G) (Tamura et al., 2021).

3. RESULTS

3.1 Microsatellite data analysis in Tench

3.1.1 Allele polymorphism and population size

All twelve microsatellite loci were successfully amplified, and all showed moderate to high levels of polymorphism, with a total of 64 alleles in all populations, 11 of which were private alleles. At polymorphic loci, the number of distinct alleles ranged from 2 (locus MTT-2) to 12 (locus MTT-9). The highest number of alleles was found in the population of Lake-Fertő at MTT-9, the most diverse site. Consequently, across all populations, the mean number of alleles per population was 3.22. Derecske (2.41 ± 1.16) and Lake-Fertő (4.16 ± 2.24) had the lowest and highest mean number of alleles, with allelic richness ranging from 2.02 ± 0.69 (in Derecske) to 3.27 ± 1.36 (in Lake-Fertő). The mean observed heterozygosity across all loci within a population ranged from 0.17 (in Derecske) to 0.47 (in Cun-Szaporca). In terms of both Ho and He, for all populations, the mean values were 0.37–0.40, respectively. Table 1 lists the basic population genetic characteristics of the stocks.

Table 1. Genetic diversity data of the seven tench populations studied.

	Lake Fertő	Lake Kolon	Csörnöc-Herpenyő	Derecske	Cibakházi Holt-Tisza	Lake Tisza	Cun-Szaporca
Na	4.16 ± 2.24	3.83 ± 1.69	3.16 ± 1.40	2.41 ± 1.16	3.25 ± 1.42	2.91 ± 1.16	2.83 ± 1.19
Neff	2.22 ± 0.78 [a]	1.81 ± 0.57 [ab]	1.92 ± 0.61 [ab]	1.38 ± 0.29 [b]	1.80 ± 0.63 [ab]	1.92 ± 0.50 [ab]	1.92 ± 0.69 [ab]
Ho	0.43 ± 0.20 [b]	0.32 ± 0.16 [ab]	0.44 ± 0.22 [b]	0.17 ± 0.13 [a]	0.37 ± 0.20 [ab]	0.36 ± 0.18 [ab]	0.47 ± 0.31 [b]
uHe	0.49 ± 0.18 [a]	0.39 ± 0.20 [ab]	0.43 ± 0.19 [ab]	0.25 ± 0.16 [b]	0.40 ± 0.18 [ab]	0.47 ± 0.17 [ab]	0.43 ± 0.25 [ab]
F	0.11 ± 0.19 [ab]	0.15 ± 0.18 [ab]	-0.03 ± 0.15 [a]	0.28 ± 0.31 [b]	0.04 ± 0.19 [ab]	0.16 ± 0.30 [ab]	-0.12 ± 0.32 [a]
AR	3.27 ± 1.36	2.74 ± 1.02	2.59 ± 1.05	2.02 ± 0.69	2.50 ± 0.93	2.78 ± 1.03	2.72 ± 1.11
AR_p	0.40 ± 0.42 [a]	0.12 ± 0.22 [ab]	0.16 ± 0.21 [ab]	0.02 ± 0.06 [b]	0.12 ± 0.24 [ab]	0.12 ± 0.31 [ab]	0.10 ± 0.29 [ab]

Na: mean number of alleles per population, *Neff*: effective number of alleles, *uHe*: unbiased expected heterozygosity, *Ho*: observed heterozygosity values, *F*: inbreeding coefficient, A_R: allelic richness, A_{Rp}: private allelic richness. If indicated, superscript letters (a, b) indicate significant differences ($p < 0.05$) between the groups.

The population of Lake-Fertő had the highest diversity, while the Derecske population had significantly lower genetic diversity. The effective number of alleles, heterozygosity (uHe and Ho), and private allelic richness were significantly lower compared to the Lake-Fertő population, and the F_{is} value was highest in the Derecske population.

Probability tests of Hardy-Wienberg showed that the MTT6, MTT8, MT3 in the Lake-Fertő population, MTT8, MTT2 in the Derecske population, MT3, MTT9 in the Lake Kolon population, MTT6, MTT8 in the Cibakházi Holt-Tisza population, and MT3 in the Cun-Szaporca population, were all found to be out of the HW equilibrium (P<0.05).

3.1.2 Population structure

Pairwise F_{ST} analysis of the microsatellite data also revealed a robust structure. The global F_{st} was 0.080 (95% CI: 0.057–0.108) with ENA correction, showing relatively modest genetic distances. By clustering 175 individuals using the STRUCTURE software (Figure 3), the most probable number of K = 4 clusters was identified. Contrary to expectations, none of the four clusters were closely related to localization within or between watersheds; only their proportions were different. The first cluster (red color) is frequent in the North-Western region (Lake-Fertő); the second (green color) in the Central-Eastern region (Lake Kolon and Derecske); the third cluster (blue color) in the Central and South-Western regions (Csörnöc-Herpenyő, Cun-Szaporca); and the fourth (yellow color) in the Eastern region (Cibakházi Tisza). The population of Tisza Lake was a mixed population with an equal frequency for all clusters (most likely due to human intervention—tench are frequently stocked in this water body). This pattern of distribution was also seen in the Principal Coordinate Analysis (PCoA). Analysis of molecular variance (AMOVA) showed that genetic variance was predominantly within the population.

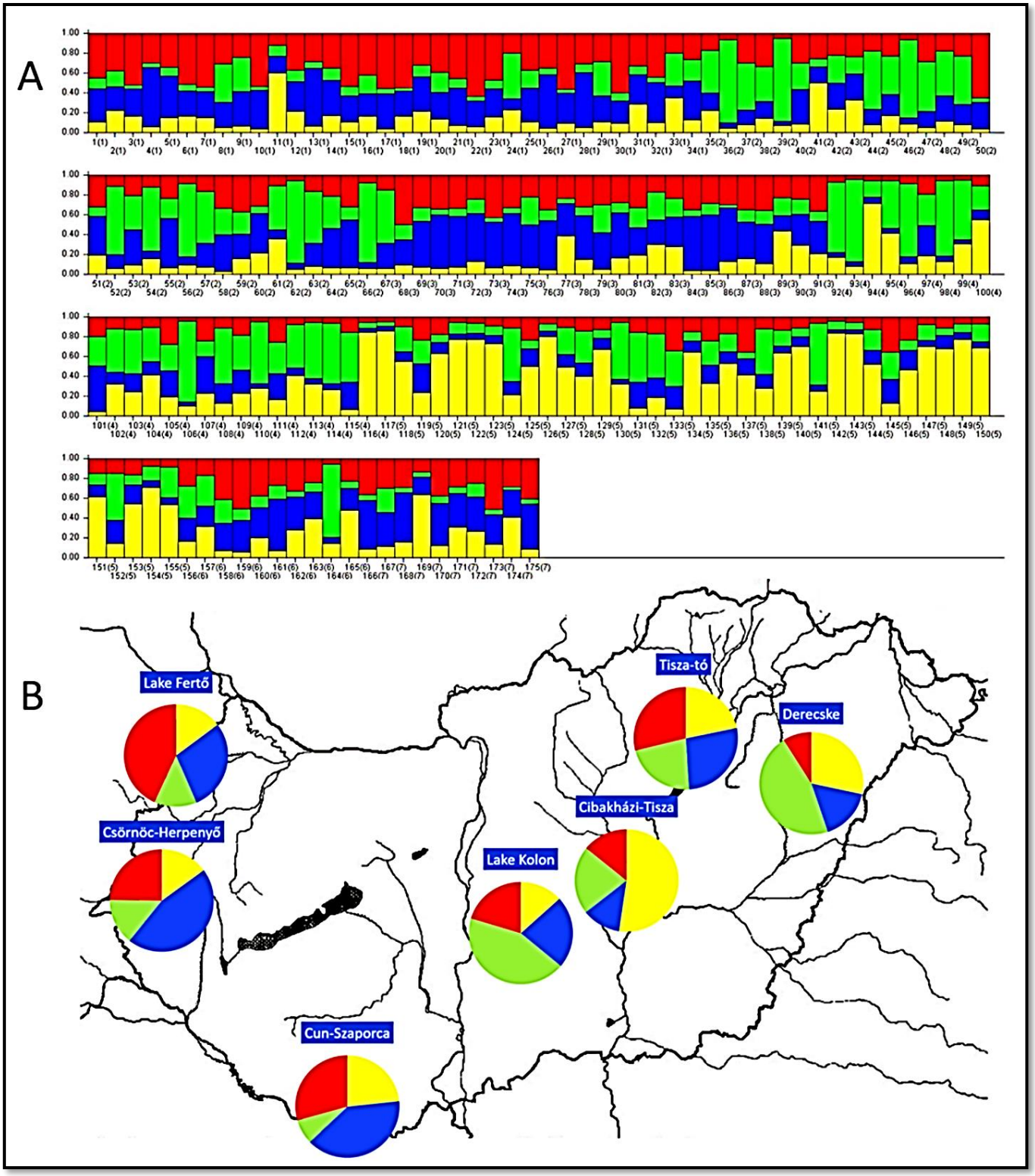


Figure 3. A Structure of the seven tench populations for K = 4, based on the microsatellite data. The populations are the following: 1st Lake Fertő, 2nd Lake Kolon, 3rd Csörnöc-Herpenyő, 4th Derecske, 5th Cibakházi Tisza, 6th Tisza-tó, 7th Cun-Szaporca. B The average contribution of the four genetic clusters in the seven tench populations.

3.2 Mitochondrial DNA analysis in Tench

The seven tench populations yielded a total of 20 new mitochondrial Cytb haplotypes, two of which were identical to sequences in GenBank (hap6- HM560230.1, HM167941.1, HM167943.1, HM167945.1, HM167946.1, HM167949.1, JX974523.1, JX974524.1, JX974525.1, and hap11-NC_008648.1:14394-15, HM167950.1, HM167952.1, JX974520.1, and JX974521.1). The samples showed a predominance of four haplotypes (haplotypes 3, 4, 6, and 11). Most of the haplotypes with small numbers of samples were found in the Lake Fertő population, which had 14 of the 20 different haplotypes. The number of polymorphic sites within the haplotype groups was 14, with 13 of them being parsimony informative.

The twenty Cytb haplotypes described in the seven populations were divided into two major haplogroups defined by Lajbner et al. (2007) and Lajbner & Kotlík (2011). An average of 28% of individuals belonged to the Western haplotype group, while 72% belonged to the Eastern haplotype group. Interestingly, 100% of the Lake Tisza individuals belonged to the Eastern haplotype. However, since the lake is stocked with tench of unknown Cytb haplotype, this information must be regarded critically, and it is not advisable to draw far-reaching conclusions from this. When we excluded the Tisza Lake population, the highest proportion of Eastern haplotypes was found in Lake Kolon (92%). Lake-Fertő had the highest proportion of Western haplotypes, with 47% of individuals belonging to this group (Figure 4, Table 2).

Table 2. Proportion of Western and Eastern haplotypes of the mitochondrial Cytb gene in seven tench populations.

	Cytb	
	Western (%)	Eastern (%)
Derecske	37	63
Lake Tisza	0	100
Cibakházi Tisza	43	57
Lake Kolon	8	92
Cun-Szaporca	40	60
Csörnöc-Herpenyő	23	77
Lake Fertő	47	53
Average	28	72

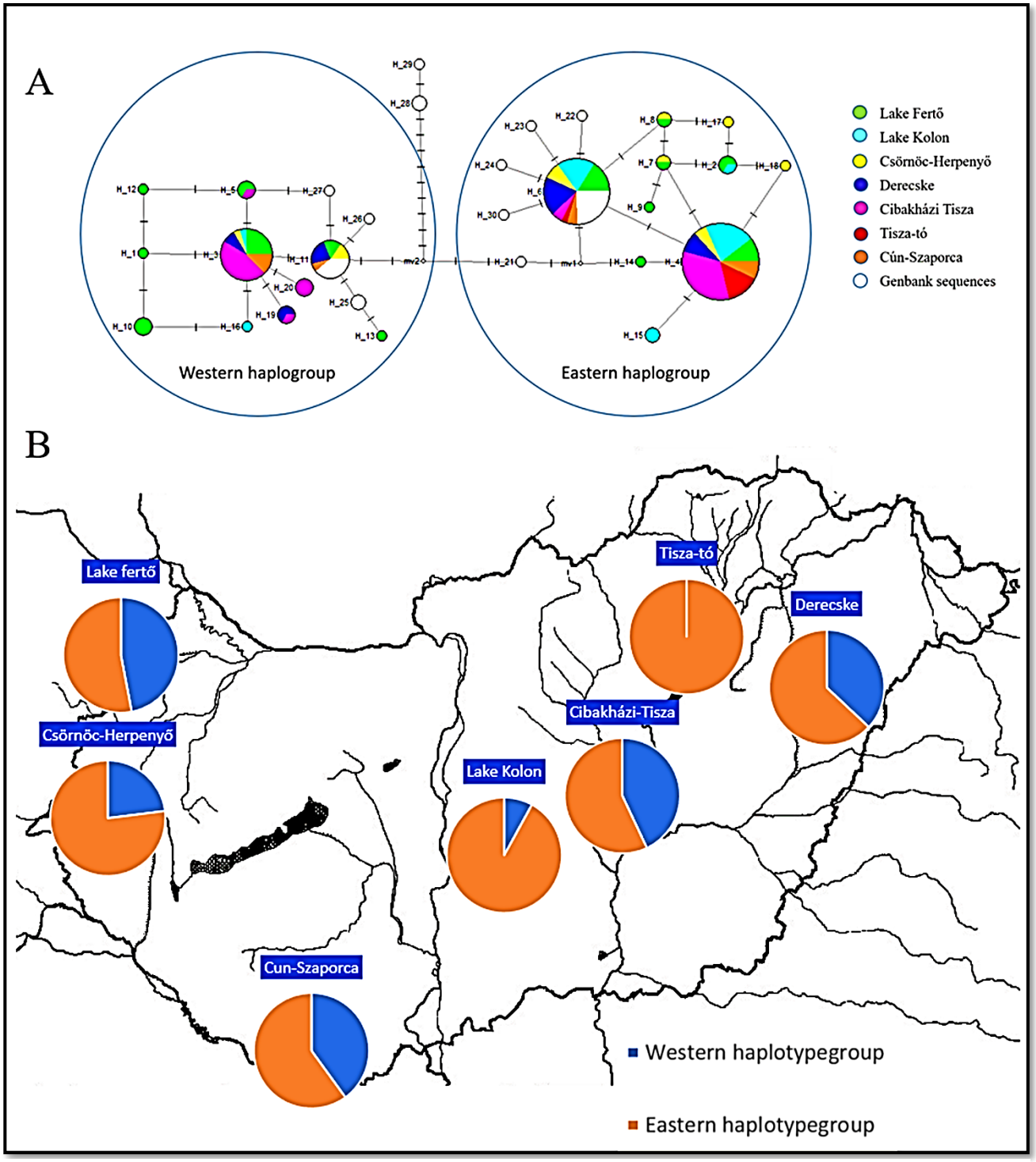


Figure 4. A. MtDNA haplotype networks for Cyt b mitochondrial DNA sequences. The size of the circles represents the number of observations of particular haplotypes. The GenBank identifiers of haplotypes described in the network figure but not found in the Hungarian samples are the following: H21: HM167942.1, H22: HM167944.1, H23: HM167947.1, H24: HM167948.1, H25: HM167951.1, JX974522.1, H26: HM167953.1, H27: HM167954.1, H28: HM167955.1, H29: HM167957.1, H30- MT605881.1. B. The relative contributions of the two haplogroups to the seven tench populations.

The NJ phylogenetic tree showed that the 20 haplotypes fell into two different phylogenetic lineages, the Eastern lineage (E) and the Western lineage (W). The first lineage was identified throughout the central and eastern Hungarian watersheds studied, while the second lineage was found predominantly in the western Hungarian catchments, mainly in Lake Fertő (Figure 5).

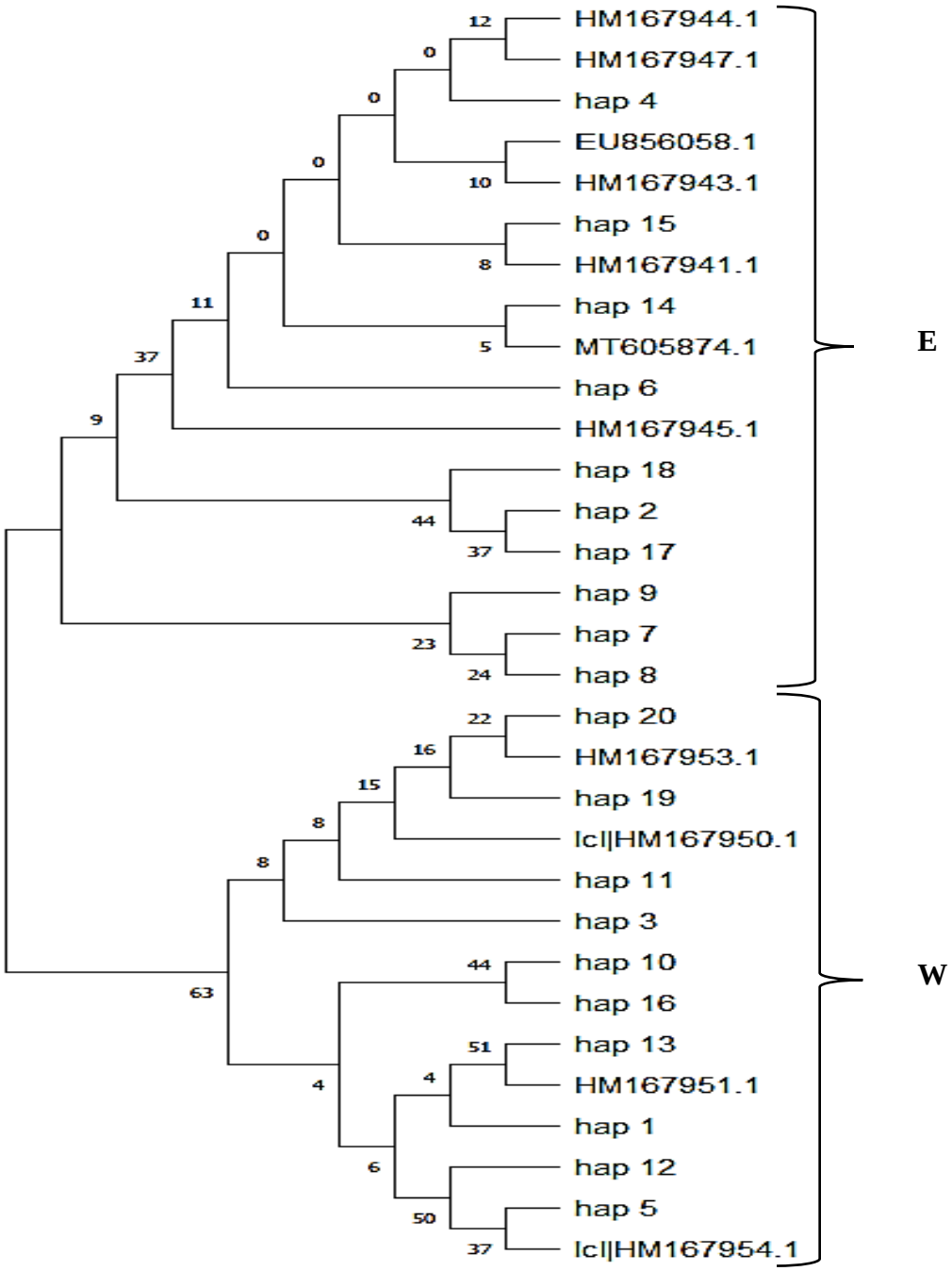


Figure 5. Neighbour-Joining tree of seven wild tench populations based on cytochrome b haplotypes. The NJ tree was constructed with MEGA-11 software using the Bootstrap method with 1,000 bootstrap replicates, Tamura 3 parameter model, Gamma-Distributed (G) (Tamura et al., 2021).

3.3 PCR-RFLP analysis of Act and RpS7 nuclear genes for phylogeography

The PCR-RFLP analysis of *Act* and *Rps7* genes showed that the proportion of heterozygote individuals was close to 50%, 41% in the case of Rps7 and 50% in the case of *Act*, respectively (Table 3). Based on both nuclear genes, an average of 33% of individuals belonged to the Western lineage, whereas 17% (*Act*) and 26% (*Rps7*) belonged to the Eastern lineage. Interestingly, in the Cun-Szaporca population, 60% of individuals belonged to the Western lineage in the case of both nuclear genes, with no individuals from the Eastern lineage present, while 40% of individuals were heterozygous. The Lake Tisza population showed the opposite picture for the two genes. In the case of the Act gene, 90% of the individuals were heterozygotes, while in the case of the *Rps7* gene, only 10% of the fish were heterozygous, and the Western lineage was dominant. The proportions belonging to each lineage were more equal in the rest of the populations studied.

Table 3. *Act* and *Rps7* haplotypes of the seven tench populations.

	Act (Eco52l)			*Rps7* (Ndel)		
	W (%)	E (%)	WE-het (%)	W (%)	E (%)	WE-het (%)
Derecske	8	36	56	4	56	40
Lake Tisza	10	0	90	60	30	10
Cibakházi Tisza	50	16	34	37	3	60
Lake Kolon	16	31	53	12	47	41
Cun-Szaporca	60	0	40	60	0	40
Csörnöc-Herpenyő	54	13	33	12	32	56
Lake Fertő	32	24	44	44	15	41
avarage	33	17	50	33	26	41

W - West; E - East; WE-het – Western-Eastern Heterozygote.

3.4 Microsatellite data analysis in Crucian carp

3.4.1 Genetic diversity and population size

All 13 microsatellite loci were found to be polymorphic in 320 individuals' genotypes. A total of 245 microsatellite alleles were described throughout the eleven natural populations and stocks of crucian carp. The lowest number of alleles was found on locus GF1 (3), while the highest number of alleles (75) was detected on HLJYJ041. The number of alleles ranged from 61 to 133 in different populations, along with a total of 49 private alleles with frequencies ranging from 0.016 to 0.250, which are mostly found in wild populations. The mean number of alleles for each population

ranged from 4.69 ± 4.35 in the farmed population (Rétimajor) to 10.23 ± 9.19 in the wild population (Cún-Szaporca Holt-Dráva), with allelic richness ranging from 4.00 ± 3.34 in the farmed (Rétimajor) population to 7.98 ± 6.12 in the wild (Mura Vízgyűjtő) population. Although wild populations had a higher allelic richness (7.25 ± 5.48) than farmed populations (5.04 ± 3.50), the difference was not statistically significant (P = 0.007) (Table 4).

The mean observed heterozygosity (Ho) and expected heterozygosity (He) for all populations was 0.509 and 0.551, respectively, and the mean observed heterozygosity across all loci within a population ranged from 0.39 ± 0.35 (HAKI) to 0.62 ± 0.24 (Alag Éger). The mean genetic parameters for eight natural populations and three stocks are shown in Table 5. The mean values of observed heterozygosity and expected heterozygosity for the eight wild populations were (0.570 ± 0.283) and (0.616 ± 0.277), respectively, whereas for the cultivated populations they were lower (0.450 ± 0.330) and (0.531 ± 0.299). In general, wild crucian carp populations had a somewhat greater level of polymorphism than cultured populations when genetic parameters were compared, including allelic richness, observed heterozygosity, and expected heterozygosity (Table 4). He and Ho values indicated that in most natural populations, these two values were close to each other. But, the Chi-square test for Hardy-Weinberg equilibrium showed a significant heterozygote deficit in six of the thirteen loci in two farmed stocks (the NBGK-HGI stock and the HAKI stock).

Table 4. Genetic differentiation of wild and cultivated crucian carp populations.

Stocks	H_O (mean ± SD)	H_E (mean ± SD)	AR (mean ± SD)	AR_p (mean ± SD)	F_{IS} (mean ± SD)
Wild	0.570 ± 0.283	0.616 ± 0.277	7.25 ± 5.48	0.49 ± 0.81	0.047 ± 0.269
Cultivated	0.450 ± 0.330	0.531 ± 0.299	5.04 ± 3.50	0.15 ± 0.36	0.209 ± 0.373
P-values	0.041	0.126	0.007	0.001	0.022

Ho = observed heterozygosity; H_E = expected heterozygosity; A_R = Allelic Richness; A_{rp} = Private Allelic Richness; F_{IS} = inbreeding coefficient; *SD* = standard deviation.

Table 5. Mean genetic parameters for eleven crucian carp populations.

Parameter	Cún-Szaporca Holt-Dráva	Dunafalva	Kölked Misányi-fok	NBGK-HGI -genebank	Lake Fertő	Lake-Kolon	Belső telep HAKI 24.	Rétimajor 21.	Baja 24.	Alag Éger	Mura-Vízgyűjtő
Na	10.23 ± 9.19	7.23 ± 6.94	7.23 ± 7.19	7.23 ± 5.93	9.00 ± 7.29	8.15 ± 7.27	4.92 ± 3.73	4.69 ± 4.35	8.15 ± 7.67	8.31 ± 5.54	9.46 ± 8.16
Ne	6.15 ± 6.85	5.13 ± 5.55	4.73 ± 5.09	3.67 ± 3.11	5.41 ± 5.49	4.58 ± 4.14	2.88 ± 2.45	2.91 ± 2.37	5.01 ± 5.03	5.12 ± 4.25	6.08 ± 6.33
I	1.47 ± 1.06	1.25 ± 1.03	1.20 ± 1.04	1.19 ± 0.86	1.45 ± 0.93	1.37 ± 0.88	0.94 ± 0.75	0.90 ± 0.84	1.31 ± 1.03	1.51 ± 0.81	1.54 ± 0.95
Ho	0.53 ± 0.31	0.52 ± 0.35	0.49 ± 0.37	0.42 ± 0.35	0.54 ± 0.24	0.56 ± 0.30	0.39 ± 0.35	0.41 ± 0.35	0.57 ± 0.35	0.62 ± 0.24	0.56 ± 0.26
He	0.58 ± 0.31	0.53 ± 0.33	0.50 ± 0.36	0.53 ± 0.31	0.61 ± 0.25	0.60 ± 0.27	0.45 ± 0.32	0.43 ± 0.36	0.54 ± 0.34	0.65 ± 0.25	0.64 ± 0.26
uHe	0.59 ± 0.31	0.54 ± 0.34	0.51 ± 0.37	0.54 ± 0.31	0.62 ± 0.26	0.61 ± 0.27	0.46 ± 0.32	0.43 ± 0.36	0.55 ± 0.34	0.66 ± 0.26	0.65 ± 0.26
F	0.09 ± 0.15	0.10 ± 0.32	0.01 ± 0.11	0.28 ± 0.36	0.08 ± 0.31	0.07 ± 0.36	0.29 ± 0.46	0.02 ± 0.17	-0.08 ± 0.24	0.02 ± 0.23	0.08 ± 0.33
A_R	7.74 ± 6.43	6.75 ± 6.24	6.01 ± 5.41	5.68 ± 4.21	7.69 ± 5.94	6.34 ± 4.92	4.21 ± 3.00	4.00 ± 3.34	6.64 ± 5.65	6.99 ± 4.34	7.98 ± 6.12
A_{Rp}	0.46 ± 0.50	0.46 ± 0.99	0.16 ± 0.42	0.15 ± 0.31	0.59 ± 0.91	0.22 ± 0.45	0.11 ± 0.39	0.15 ± 0.38	0.60 ± 1.20	0.75 ± 0.84	0.55 ± 0.82

Na = mean number of alleles per population; *Ne* = mean number of effective alleles; *I* = Shannon's index; *Ho* = observed heterozygosity; *He* = expected heterozygosity; *uHe* = unbiased expected heterozygosity; *F* = inbreeding coefficient, A_R = allelic richness; A_{Rp} = private allelic richness.

3.4.2 Population structure and genetic differentiation

Pairwise F_{st} analyses were used to illustrate the patterns of genetic divergence among crucian carp populations across all loci. Without ENA correction and with it, the global Fst was 0.226 (95% CI: 0.137-0.340) and 0.217 (95% CI: 0.131-0.326) respectively, showing that the eleven populations studied had moderate to high genetic distances. Clustering the eleven crucian carp populations by STRUCTURE software resulted in the most probable number of two clusters, K = 2. One distinct cluster was classified as "natural populations," while the other cluster was classified as farmed stocks. Three of the eleven populations, NBGK-HGI, HAKI, and Rétimajor, were grouped into the cluster of farmed stocks, suggesting that they had different origins. Further STRUCTURE analysis of the eight wild populations was performed to determine the most probable K (K = 3), which showed that populations are mainly clustered into three sub-populations within the eight native populations (Figure 6A). The uniformity or mixing of the colors defined the population's genetic structure. For each sample site, the presence of just one color implies a population with no admixture; the presence of two or more colors shows genetic admixture at that particular sample site (Figure 6B). This analysis confirmed the genetic variability of the crucian carp populations. The two natural populations, Dunafalva and Kölked (close to the Danube River), were genetically homogenous, with no genetic admixture. These are completely isolated pond populations with a small number of individuals, most likely as a result of a prior occurrence of a founder effect. The populations from the Danube region, Lake-Kolon, Baja, Alag, and Cún-Szaporca Holt-Dráva from the Drava region, showed relatively minimal genetic admixture, suggesting fewer contributions or mixing with other populations. Although there was no significant association between the geographical distance of the wild populations of Lake-Fertő, Mura and some other populations, these populations showed a great deal of admixture in terms of population structure. It was found that these populations share some clusters, indicating gene flow or mixing across the populations, either naturally or by the introduction of fish from other populations where gene flow has occurred. Individual relationships within and between groups were also evident using Principal Coordinate Analysis (PCoA) (Figures 6C). The analysis of molecular variances (AMOVA) revealed that 20% of the total molecular variance was among populations, 16% was among individuals, and 64% was within individuals, with a significant level (P<0.001).

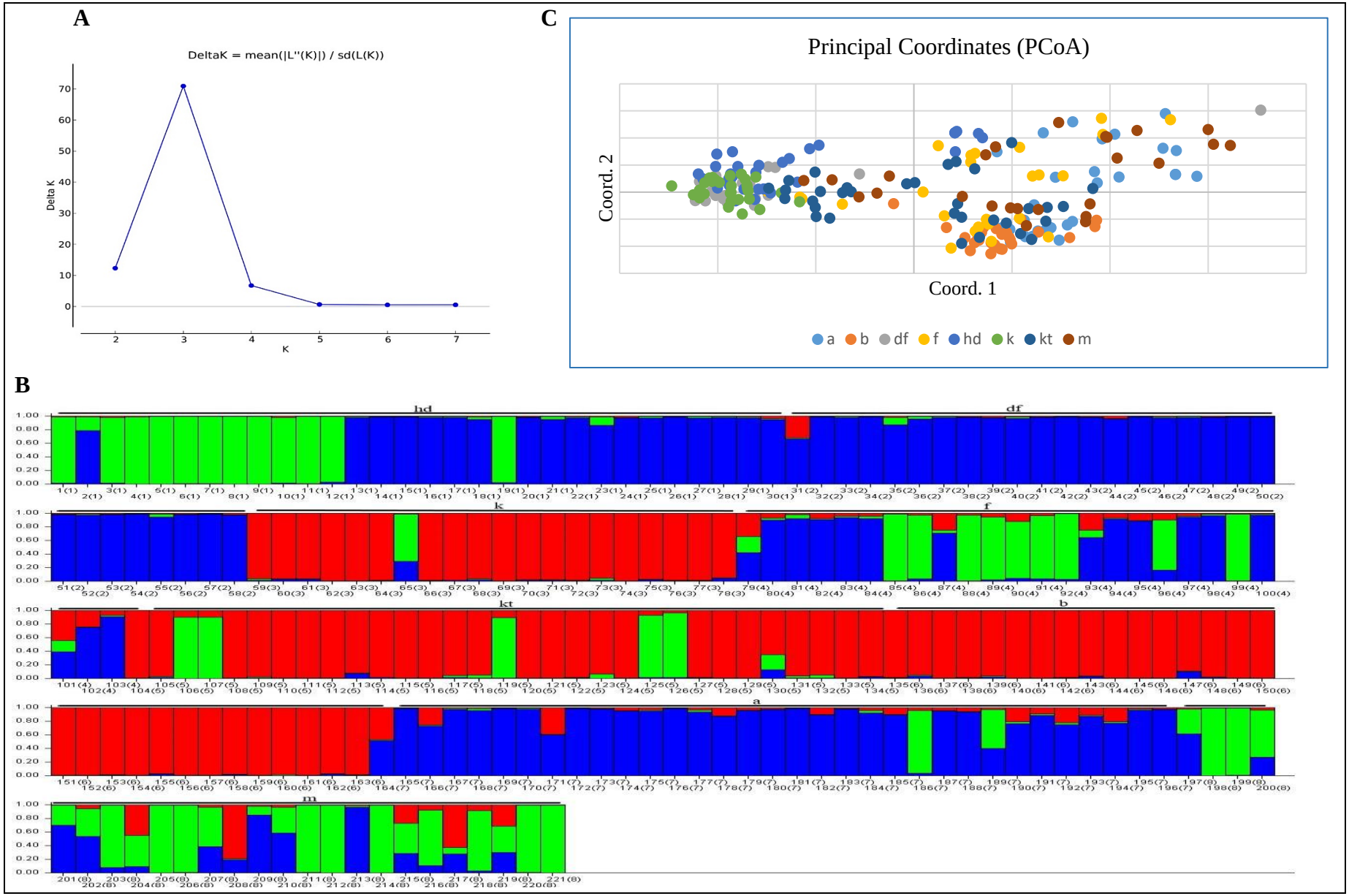


Figure 6. Population structure results for eight natural crucian carp populations: A Best delta K estimation. B Bayesian clustering analysis of eight wild crucian carp populations using STRUCTURE v2.3.3. Three clusters (k = 3) were inferred, indicating the presence of three distinct subpopulations of crucian carp in Hungary. In this plot, these sub-populations are represented by three colors: green, red, and blue. Each vertical line represents one individual. A population of uniform color (i.e. either green, red or blue) represents a population with little or no admixture. However, admixture is present in most populations in the current study. The only two exceptions are population 2 (df) and population 3 (k). C Principle Coordinate Analysis (PCoA) of allele frequencies in eight wild crucian carp populations.

3.5 Phylogenetic analysis of Crucian carp Based on the COI gene sequence

To find out the phylogenetic relationship between the crucian carp (*Carassius carassius*) and the silver crucian carp (*Carassius auratus gibelio*), the COI haplotypes of *Carassius carassius* and *Carassius gibelio* from GenBank were compared to the new *Carassius carassius* COI haplotypes found in this study. 104 COI sequences yielded a total of 47 haplotypes, 43 of which were novel and four were previously described (Figure 6). The number of polymorphic sites within the haplotypes was 90. The NCBI database was employed to verify the sequences' evolutionary origins. After being blasted, six haplotypes (hap6, hap20, hap21, hap22, hap23, hap24) were found to be those of silver Prussian carp (*Carassius auratus gibelio*), while the rest were identified as crucian carp (*Carassius carassius*). Thus, the haplotypes found showed two main groups in the network diagram (Figure 7).

The Neighbor-Joining phylogenetic tree revealed that the sequences were divided into three separate clusters, one of which included the *Carassius auratus gibelio* sequences, which were morphologically identified as crucian carps. The six gibel carp haplotype sequences identified among the new sequences, together with the 14 gibel carp haplotype sequences described in GenBank, formed the first cluster (A). The hap24, which was assigned to *Carassius auratus gibelio* in the network, was found to be separated from the cluster (A). The second cluster (B) included four *Carassius carassius* sequences from GenBank as well as individuals from rm, ha, and gb stocks, confirming the results of the microsatellite analysis and highlighting the different origins of the farmed stocks. The remaining haplotypes of individuals from all locations were represented in the biggest cluster (C), which was identified as a *Carassius carassius* species supported by two *Carassius carassius* sequences from the GenBank (Figure 8). These results show that gibel carp have invaded crucian carp populations and that the two species have taken different paths in their evolution.

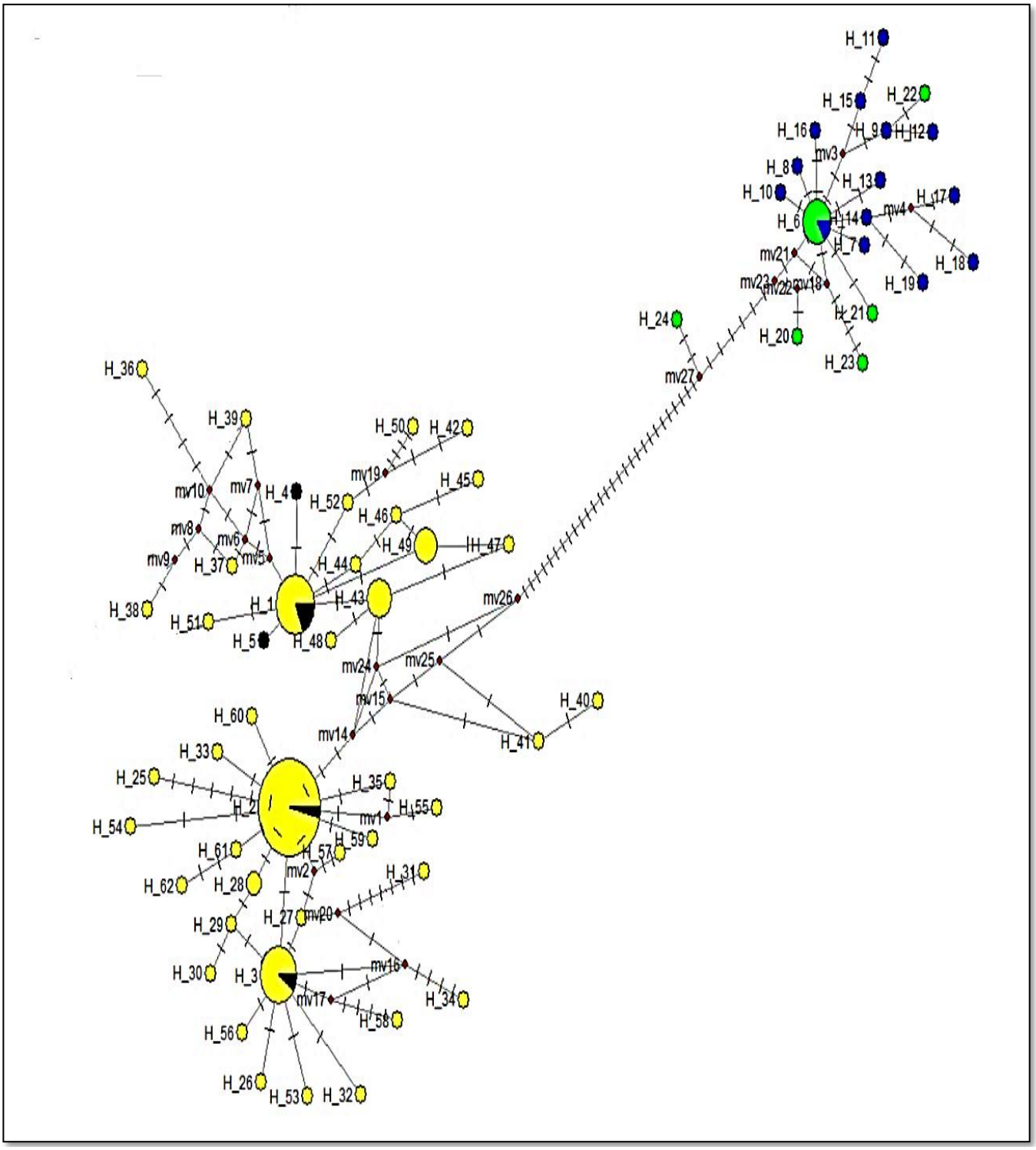
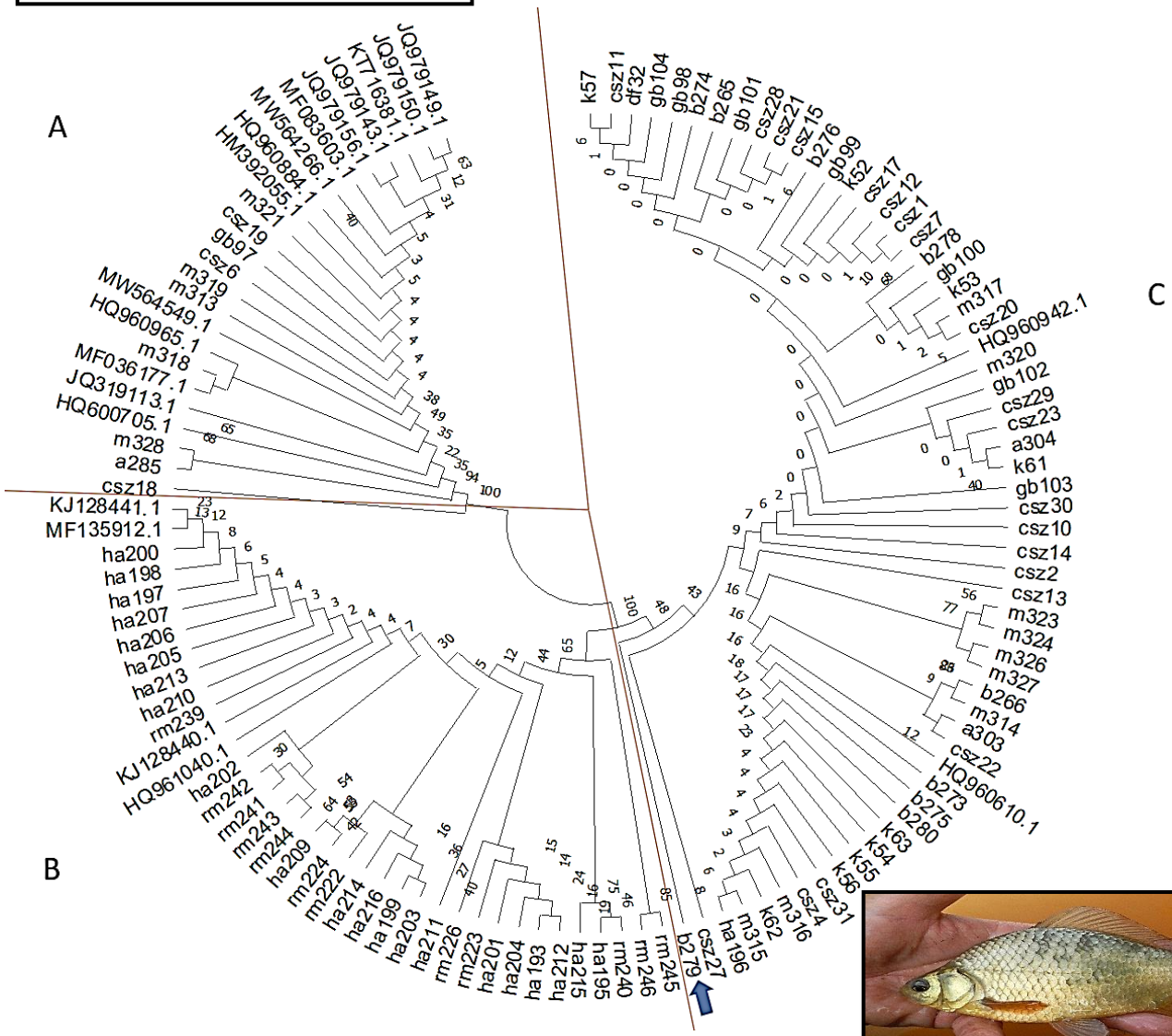


Figure 7. Haplotype networks of COI mitochondrial DNA sequences, showing the relationship between native crucian carp and closely related gibel carp species. Each circle represents a haplotype, and the sizes of the circles indicate the frequencies of each haplotype. Yellow, crucian carp; Green, gibel carp; Black, GenBank reference sequences. The median vector with red dots represents a predicted haplotype that was not identified.

Carassius gibelio



Carassius carassius

Figure 8. Neighbour-Joining tree of genetic distances between *Carassius carassius* and *Carassius gibelio* based on COI mtDNA sequences. Cluster A represents *Carassius gibelio*, clusters B and C represent *Carassius carassius*. The blue arrow indicates the separation of haplotype 24 (see Network) from the three clusters. The NJ tree was constructed with MEGA-11 software using the Bootstrap method with 1,000 bootstrap replicates, Tamura 3 parameter model, Gamma-Distributed (G) (Tamura et al., 2021).

4. NEW SCIENTIFIC RESULTS

1. The population genetic structure of tench (*Tinca tinca* L. 1758) and crucian carp (*Carassius carassius* L. 1758) reported in this study supported previous studies from different geographical regions in Europe. We were able to confirm the combined use of nuclear and mitochondrial markers as an effective way to better understand the genetic makeup of a population since each contributes unique features. For both species, we applied a larger number of microsatellite markers than had been used in most previous studies.

2. This study is the first to describe the genetic diversity and population structure of tench (*Tinca tinca* L. 1758) populations in the Carpathian Basin.

3. The current study revealed that, in addition to the sequencing of an mtDNA (Cytb) segment, the PCR-RFLP analysis of two independent nuclear-encoded exon-primed intron-crossing (EPIC) markers, (*Act*) and (*RpS7*), is an efficient method for assessing phylogeographic structure and identifying the Western and Eastern origins of Hungarian tench populations.

4. A total of twenty Cytb haplotypes from Hungarian tench samples were described in this study. Of these, 18 (including the common H3 and H4) had not been previously observed.

5. Based on COI gene data, we provide the first phylogenetic and taxonomic overview of native crucian carps and the closely related invasive gibel carps found in Hungarian waters. A total of 47 COI haplotypes were identified, with 43 of them being novel. Six of the new haplotype sequences were identified as belonging to *Carassius gibelio*, confirming previous reports of their invasion across different parts of Europe.

5. CONCLUSIONS AND PERSPECTIVES

In this thesis, the population structure and diversity of endangered crucian carp (*Carassius carassius* L. 1758) and tench (*Tinca tinca* L. 1758), as well as phylogenetic relationships and systematic phylogeography, are explained using genomic approaches, paving the way for the accurate identification of Hungary's conservation management units and selective breeding programmes. The following are the main conclusions:

- A key consideration in species conservation is to protect and maintain genetic variability, as described in the literature section above. This part of the thesis revealed that the wild living populations of tench in Hungary are genetically moderately diverse compared to other natural populations of the species living in Western Europe, and this is the first study in which genetic diversity in native Hungarian tench populations was described. However, they still represent significant aquatic genetic resources and could serve as a good basis for future selective breeding programmes. The genetically most variable Lake Fertő, Lake Kolon and Csörnöc-Herpenyő populations can be the most promising candidates for future breeding programs, while populations with considerably high private allelic richness (such as Cibakházi-Tisza and Lake-Tisza) should also be involved in order to start such a program with the highest genetic variability involved.

  This part of the thesis also showed that there are moderate to high genetic differences in the populations of Hungarian crucian carp, which is seen as a first initiative in assessing the genetic diversity and population structure of Hungarian aquaculture stocks. The natural populations, on the other hand, had higher variability. Two wild populations, Dunafalva and Kölked (close to the Danube River), were found to have minimal genetic diversity and a homogeneous genetic structure, perhaps owing to the presence of a founder effect in these isolated pond populations. The ponds that don't have much or any mixing have a good chance of becoming future stocks for the native crucian carp populations.

  To ensure the survival of the population and its ability to adapt to environmental change, a wide range of genetic variations is necessary. Wild populations are essential genetic resources, and therefore it is crucial to preserve them. We recommend monitoring the genetic diversity of the farmed stocks of these species in order to keep biodiversity from being lost. Genetic variation in stocks can be more effectively monitored with the use of

molecular approaches. This helps us understand how this diversity can be maintained through selective breeding. Appropriate breeding practices, as with crossbreeding can be used to enhance genetic variety in farmed stocks having limited genetic variation. However, uncontrolled crossbreeding, must be avoided. Additionally, increasing effective population sizes will help reduce inbreeding levels, which must be considered while developing breeding programs for these species. In the future, the research will be expanded to include more wild populations from a wider geographic area and more cultivated stocks so that the genetic profile of the populations found in this study can be tracked.

- Tench's natural range is also an important consideration. In this part, the phylogeographic analysis described the Western and Eastern phylogroups and was able to find hybrids between the two clades. Thus, Hungary is in the transition zone between the two lineages of the species, with a high level of hybridization, suggesting that these phylogroups were dispersed outside of their native range through human activities. This interrogation will help to distinguish the diverse geographic origins of populations and track the phylogroups spread across wild populations mediated by humans, which is important for breeding efforts. However, to learn more about this phenomenon, the natural and human-aided processes of hybridization in this zone need to be studied in more depth.
- We found indications of invasive *Carassius auratus gibelio* extension in Hungarian crucian carp populations and assessed their phylogenetic relationship. Mitochondrial data has provided useful insights in resolving taxonomic ambiguities among closely related species that are difficult to define morphologically. The Cytochrome C Oxidase I (COI) gene was shown to be useful in identifying the phylogenetic relationships between *Carassius carassius* and the closely related invasive gibel carp, *Carassius auratus gibelio*. The distribution of haplotypes indicated that these two species had diverged in their evolution. Therefore, although most of the hybrids exhibited morphology similar to *Carassius carassius*, *Carassius carassius* populations should be treated as separate management units for conservation activities. However, the current understanding of the taxonomic status of the two species provided by these data is still insufficient. Therefore, more specimens will be included in the next step of our studies to precisely define their taxonomic position in the *Cyprinidae* family. On the other hand, the invasion of species gives us the opportunity

to consider the many different evolutionary processes that occur within species. This result encourages further research into the impact of invasive species on the genetic diversity of Hungarian native species.

- The National Centre for Biodiversity and Gene Conservation has established an ex-situ live GenBank and a cryobank to help achieve the study's intended aims. Ex-situ conservation practices must emphasize the genetic structure and identity of translocated individuals.

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7. LIST OF PUBLICATIONS

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1-Q1; impact factor: 2.465

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F. Al-Fatle Ali Abdulhur, E. Edviné Meleg, K. Ihász, T. Molnár, B. Kovács, I. Lehoczky (2019) Conservation of the native Cyprinid species in Hungary – preliminary results on the genetic variability of Crucian carp (*Carassius carassius* L. 1758) and Tench (*Tinca tinca* L. 1758) populations and stocks. Aquaculture Europe 19. Berlin, Germany, 2019.10.07.-10. Book of Abstracts p: 54-55.

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Al Fatle Fatema Ali, Tamás Molnár, Erika Edviné Meleg, Gergely Szabó, Gábor Fekete, Zoltán Sallai, Balázs Kovács and István Lehoczky (2021) Genetic Analysis of seven natural populations of Tench (*Tinca tinca L. 1758*) in Hungary – establishment the biological basis of selective breeding. Aquaculture Europe, Madeira, 2021.10.04.-07. Book of Abstracts p: 350-351.

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