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Phylogenetic characteristics of selected European huchen (*Hucho hucho* L.) broodstocks – implication for broodstock management

by

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Abstract

European huchen (*Hucho hucho*) is a representative of large and rare migratory salmonid fish, which has become endangered due to extensive anthropogenic changes in freshwater ecosystems. Numerous broodstocks of the European huchen have therefore been established throughout the species' range in recent years to supplement wild fisheries of this species. Unfortunately, this conservation management strategy entails a number of potential ecological and genetic risks associated with the release of farm-raised fish into wild populations. A comprehensive and feasible genetic monitoring protocol for broodstocks maintained for the production of restocking material is therefore essential in the sustainable management of critically endangered fish species. The current paper presents phylogenetic characteristics of four selected huchen broodstocks across Central and Eastern Europe. Genetic comparisons of the studied broodstocks were based on ten microsatellite DNA markers. The effective population size (*Ne*), the individual assignment test, the Principal Coordinates Analysis (PCoA), the allele sharing distance (DAS) and the Bayesian clustering analysis were applied in this study. Moreover, five selected fragments of mitochondrial DNA were used for molecular verification of species membership and genetic purity of examined specimens.

Key words: European huchen, conservation, microsatellite loci, mitochondrial DNA, phylogenetic characteristics

1. Introduction

Salmonids are both an economically and ecologically important as well as biologically interesting fish family. This group of fish is characterized by high environmental requirements, inhabiting almost all habitats with clear, cold and well-oxygenated waters. Only a few genera of Salmonidae are exclusively freshwater, while all other species are anadromous, having remarkable homing abilities, typically migrating from seawater to freshwater for spawning (Kottelat & Freyhof 2007; FishBase 2020). The extensive anthropogenic changes in freshwater ecosystems observed in recent years related to damming, industrial and agricultural pollution of the aquatic environment, overfishing, poaching and commercial fisheries have rendered many salmonid species endangered (IUCN 2020). Furthermore, inadequate fishery supplementation for recreational and commercial purposes resulted in irreversible disappearance of many native Salmonidae populations. Therefore, restitution programs have been launched in Poland in recent years to actively protect the most endangered salmonid fish species. The flagship species in Polish waters are whitefish (*Coregonus lavaretus*), Atlantic salmon (*Salmo salar*), sea trout (*Salmo trutta*), European grayling (*Thymallus thymallus*) and European huchen (*Hucho hucho*), the modern conservation management of which is mainly based on stocking strategies. Therefore, numerous broodstocks of the aforementioned fish species have been established to supplement fisheries of their wild populations. Unfortunately, this raises a number of potential ecological and genetic risks associated with the release of farm-raised fish into wild populations (Einum et al. 2001).

Determination of phylogenetic relationships between farmed fish stocks or wild populations enables high-resolution assessment of their genetic structure and differentiation as well as identification of significant genetic clusters. One of the major concerns in conservation management is inappropriate mixing of fish stocks and introduction of non-native fish to natural populations. It is considered that breeding programs, reintroductions, supplementation or elimination of migration obstacles without any genetic structure data may contribute to the extinction of already endangered fish species (Vrijenhoek 1998; Frankham et al. 2010; Geist 2011). Genetic knowledge together with available information on the species biology can be a powerful tool for effective conservation of critically endangered fish to prevent these threats. Moreover, genetic cluster recognition is essential for the identification and establishment of management units (MU), which are a necessary element of any well-designed restitution program. Such an approach to conservation management can greatly assist in the identification of distinct population units within wild populations by showing to what extent wild-caught individuals are of hatchery origin and how many interbreeding events occurred between hatchery and wild-caught stocks. Furthermore, many examples proved that the conservation and management of endangered fish species can greatly benefit from knowledge about phylogenetic characteristics of fish stocks (Wenburg et al. 1998; Hansen 2002; Geist et al. 2009; Bernas et al. 2014).

Based on microsatellite DNA data, numerous methods of genetic distance assessment as well as probabilistic assignment to genetic clusters have been developed and applied to phylogenetic analysis of fish populations and broodstocks. Genetic parameters, such as genetic distance (*Da*) (Nei at al. 1983), genetic differentiation (*Fst* and *Rst*) (Weir & Cockerham 1984; Slatkin 1995), allele sharing distance (*DAS*) (Bowcock et al. 1994) as well as assignment tests (Pritchard et al. 2000; Piry et al. 2004; Kalinowski et al. 2007), are widely used in such genetic studies. The results of such calculations are usually presented in the form of matrix data that are difficult to interpret. Therefore, specialized methods of graphic interpretation of obtained data, e.g. Principal Coordinates Analysis (PCoA), Factorial Correspondence Analysis (FCA), UPGMA (unweighted pair-group method using arithmetic averages), Neighbor-Joining (NJ) and Bayesian clustering (BA) have been developed. Since each type of genetic input data requires a different approach to phylogenetic assessment, such studies should incorporate different methods of analysis to properly characterize genetic structure and differentiation of fish groups under study.

The main objective of the present study was to present phylogenetic characteristics of farmed broodstocks of the European huchen from Poland, Germany, Slovakia and Ukraine on the basis of multiple approaches to microsatellite data analysis. In addition, five fragments of mitochondrial DNA markers were also used in the course of the present study to examine the genetic status of the studied European huchen broodstocks.

2. Materials and methods

A total of 135 specimens of the European huchen were non-invasively sampled from four isolated broodstocks. Small fin clips were obtained in

2011–2013 from fish farms located in Poland (*N* = 30; Restocking Center and Trout Hatchery Lopuszna), Germany (*N* = 32; Fish farm Linbergmuehle, Bavaria), Slovakia (*N* = 44; Fish farm Pribovce, Martin Province) and Ukraine (*N* = 28; Fish farm "Iskhan" Baniliv, Chemivtsi Province). Collected fish tissues were placed in Eppendorf tubes and kept in 96% ethanol at a temperature of 4°C until DNA extraction. The standard Chelex 100 procedure was employed to isolate genomic DNA from collected fin clips (Walsh et al. 1991).

Ten microsatellite loci: *BleTet-9*, *BleTri-2*, *Hljz-056*, *Ogo-2*, *Omm-1032*, *Omm-1077*, *Omm-1088*, *Sfo-18*, *Sfo-262* and *Ssa-197* were used for genetic screening of the selected European huchen broodstocks under current study. Furthermore, five fragments of mitochondrial DNA (mtDNA) genes: *NADH-1* (*ND1*), *NADH-5* (*ND5*), *ATPase6* (*ATP6*), *Cytochrome b* (*Cytb*) and *D-loop* (*CR*) were used for molecular verification of species membership and genetic purity of examined specimens. Amplification of selected microsatellite and mitochondrial DNA fragments was conducted using primer sets and conditions described by Kucinski et al. (2015a,b).

Forward 5'-labeled primers with various fluorescent reporter dyes (6-FAM, VIC, PET and NED) were used to carry out the genotyping of selected microsatellite DNA markers. Determination of allele profiles, expressed as lengths of amplified DNA fragments, within each microsatellite loci was performed using an Applied Biosystems Genetic Analyser 3130 sequencer against the GeneScan 600 LIZ size standard (Applied Biosystems, California, USA). The same equipment was used for sequencing the selected mitochondrial DNA fragments. Prior to that, amplified DNA templates were subjected to purification by the DNA Cleanup Kit (A&A Biotechnology) and then used for sequencing PCR reactions. All sequencing reactions were prepared using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, California, USA) following the manufacturer's protocol with minor modifications related to the reduction of the total reaction mixture volume to 10 µl and dilution of the Ready Reaction Mix to final concentration 0.5×. Finally, the amplified, fluorescently labeled and terminated DNA was salt-precipitated with the BigDye XTerminator™ Purification Kit (Applied Biosystems, California, USA) and sequenced.

The obtained microsatellite DNA raw data on individual genetic profiles of each of the studied specimens of the European huchen were processed and compiled in Microsoft Excel. The genotypic data were then checked for microsatellite null alleles, inconsistent values, scoring errors and large-allele dropout in the samples using Micro-Checker software version 2.2.3 (Van Oosterhout et al. 2004). A set of data analysis approaches was applied for genetic comparisons of the European huchen broodstocks under study and genetic cluster recognition. First, the effective population size (*Ne*) was calculated for each examined broodstock using NeEstimator software version 2.01 (Do et al. 2013). The linkage disequilibrium method was employed to compute *Ne*, where the lowest allele frequency used was 0.02. The calculated *Ne* values were subsequently corrected for underestimation due to sampling errors with 95% jackknife confidence intervals (CIs). The genetic heterogeneity of the studied broodstocks was then tested using ONCOR software version 2.0 (Kalinowski et al. 2007). For this purpose, the leave-one-out method and the individual assignment test were used. A threshold value at $p < 0.1$ was selected for broodstock assignment. If the probability of assignment is lower than the defined threshold, a given specimen cannot be definitely assigned to a specific broodstock. The Principal Coordinates Analysis (PCoA) was performed on the basis of individual pairwise genetic distances using GenAlex software version 6.5 (Peakall & Smouse 2012). Furthermore, the allele sharing distance (DAS) method was applied to assess the genetic relationships between the studied broodstocks of the European huchen (Bowcock et al. 1994). The obtained pairwise genetic distance matrix, calculated as the proportion of shared alleles at each locus, was used to construct a neighbor-joining tree of individuals with Populations software version 1.2.32 (Langella 2002). Finally, the Bayesian clustering analysis implemented in Structure version 2.3.4 (Pritchard et al. 2000) was also performed to estimate the most likely number of genetic clusters (*K*) within the studied broodstocks. *K* was tested from one to 10 with 20 iterations. The admixture model (Falush et al. 2003) was used with 20 000 burn-in periods and 1 000 000 Markov chain Monte Carlo (MCMC) replicates in each run. The most likely number of genetic clusters was estimated by the Δ*K* method (Evanno et al. 2005). Furthermore, the obtained partial sequences of *ND1*, *ND5*, *ATP6*, *Cytb* and *CR* were compared to sequences deposited in the NCBI gene bank using the Basic Local Alignment Search Tool software (BLAST, NCBI-NIH).

3. Results

Comparative analyses of the five sequenced fragments of mtDNA genes against the NCBI GenBank dataset confirmed that the examined specimens belong to the species *Hucho hucho*. Moreover, no

indications of introgression or other genetic impurities caused by interspecific hybridization were found.

The estimated effective population size (*Ne*) values for the investigated fish from Poland, Germany, Slovakia and Ukraine were: 27.7 (95%, CI = 13.5–96.8), 17.6 (95%, CI = 12.1–26.6), 7.0 (95%, CI = 3.9–10.1) and 16.2 (95%, CI = 6.5–62.4), respectively. The assignment test showed that 85.2% of the examined specimens were correctly assigned to their baseline broodstocks. The German and Slovakian broodstocks showed the best assignment with an accuracy of 100.0% of correct fits. On the other hand, the lowest match was determined for the Ukrainian broodstock (69.0%) and the largest misidentification was determined for the Polish broodstock (31.0%). The average likelihood (AVG) values confirmed these results (Table 1). The assignment test indicated an extreme misidentification only in the Ukrainian broodstock, where just one individual showed probability values lower than 0.1. The Principal Coordinates Analysis (PCoA) revealed three-dimensional relationships between all specimens of the European huchen studied (Fig. 1). The resulting scatter plot of pairwise genetic distance analysis revealed three main genetic groupings: (1) the first one comprised fish from Poland and Ukraine, while (2) the second and (3) the third grouping included individuals from the two remaining broodstocks from Germany and Slovakia, respectively. On the other hand, the constructed tree of individuals based on DAS genetic distances showed four well-established clades, where only one individual was incorrectly assigned (Fig. 2). The obtained results show that the fish from Poland and Ukraine formed one main clade. The second identified clade was composed of fish from the German broodstock, while the two remaining clades occurred within the Slovakian broodstock. Similar to the results obtained with the PCoA approach, the German and Slovakian broodstocks together were clearly differentiated from the Polish and Ukrainian group. On the other hand, the results obtained from individual multilocus genotype analyses distinguished only two genetic clusters within the studied specimens of the European huchen. The Δ*K* calculation method applied for each of the estimated log probability of data Pr(X/K) revealed the highest values at *K* = 2 (Fig. 3). The first identified genetic cluster comprised fish from Poland and Ukraine, while the second cluster encompassed the two other European huchen broodstocks (Slovakia and Germany; Fig. 4).

Table 1

Results of assignment tests for four examined European huchen broodstocks

Broodstock	Leave one method		Individual assignment	
	N	% correct	LM	AVG
Poland	30	73.3	31.0	0.8255
Germany	32	100.0	0.0	0.9998
Slovakia	44	100.0	0.0	0.9999
Ukraine	29	69.0	26.7	0.9242

N – number of individuals, % correct – ratio of correct assignments to baseline broodstocks in the leave-one-out test, LM – largest misidentification in each broodstock, AVG – average probabilities for all specimens in the mixture set belonging to each predefined broodstock.

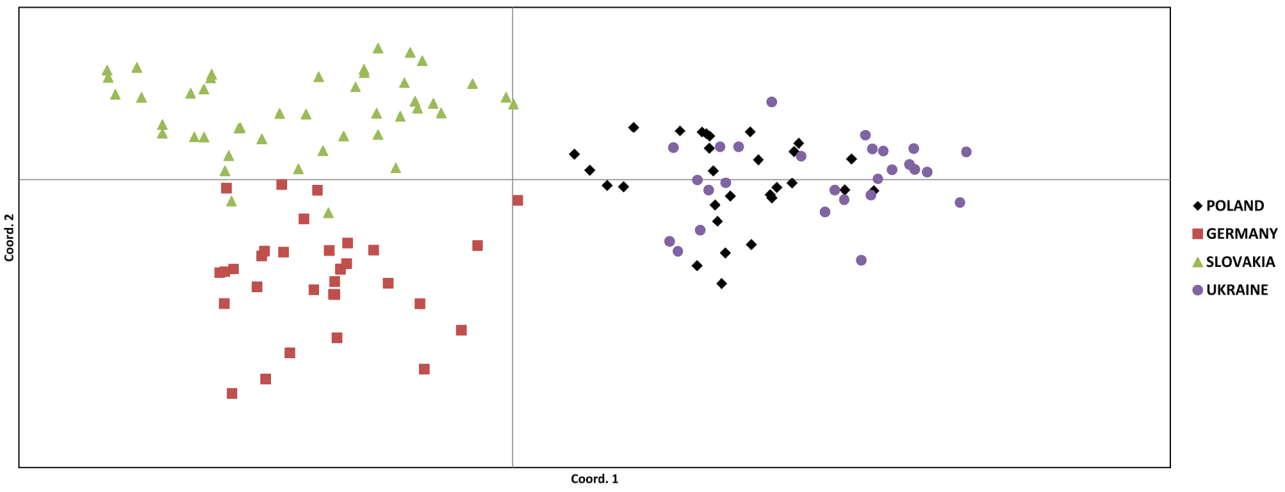

Figure 1

Scatter plot of Principal Coordinates Analysis (PCoA) based on individual pairwise genetic distances for the studied broodstocks of the European huchen

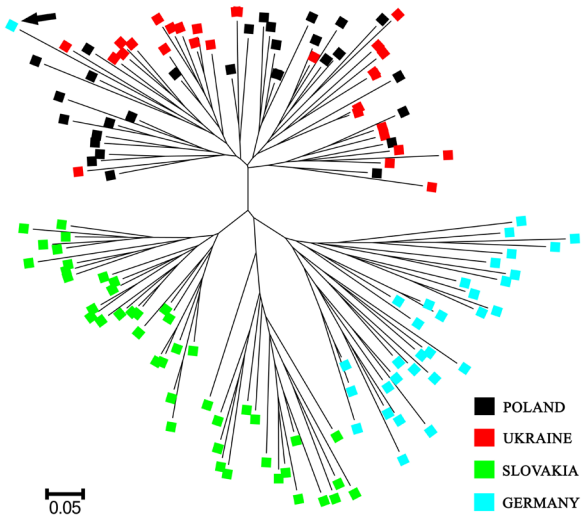

Figure 2

Unrooted neighbor-joining tree of individuals based on allele sharing distances (DAS). The arrow indicates one individual that was included in a cluster not matching its origin.

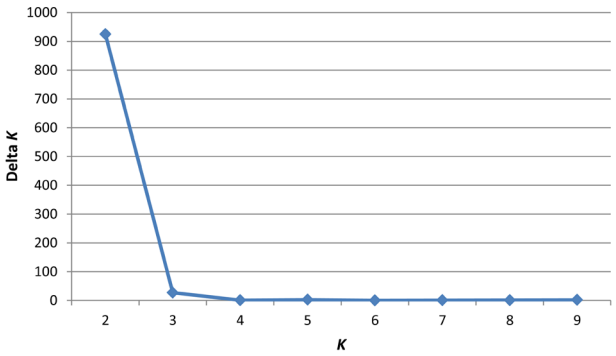

Figure 3

Plot of the rate of change in the log probability of data between successive *K* values (Δ*K*). The most likely number of genetic clusters was determined for the studied European huchen broodstocks based on the Δ*K* method.

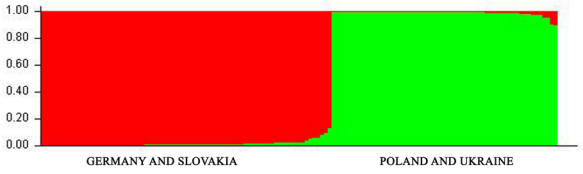

Figure 4

Structure plot of individuals sorted by admixture coefficients (q) for putative *K* = 2. Each vertical bar denotes one individual.

4. Discussion

One of the most important factors determining the efficiency of conservation management of endangered fish species is the origin and quality of stocking material. Proper identification and selection of source populations for the establishment of broodstocks and the production of stocking material is crucial in well-designed restitution programs. In addition, hatchery fish stocks are frequently subjected to altered selection pressures and many studies provided evidence that aquaculture conditions carry the risk of adverse changes in their gene pool, which contributes to the loss of genetic diversity, decreased heterozygosity, inbreeding, or outbreeding depression (Waples 1999; Wang et al. 2002; Glover et al. 2004; Wedekind et al. 2007; Huff et al. 2011). Fishery supplementation of wild populations using fry affected by inbreeding and characterized by low genetic diversity can significantly reduce the effectiveness of implemented conservation measures. Therefore, genetic monitoring of endangered fish stocks should be continuously conducted to improve the existing restocking programs. Unfortunately, due to insufficient employment or frequent lack of routine application of genetic data analysis during development of restitution programs for ecologically and economically valuable fish species in Poland, most of them have resulted in unsatisfactory outcomes. Examples include Atlantic salmon and sea trout, for which no sufficient genetic data were available, which prevented proper selection of source populations for the production of stocking material (Drywa et al. 2013; Pocwierz-Kotus et al. 2015). Therefore, an efficient and feasible genetic monitoring protocol for broodstocks maintained for the production of stocking material is a key factor determining the sufficient effectiveness of conservation management of critically endangered fish species. Genetic monitoring of broodfish should involve molecular verification of species membership and purity, broodstock genetic variation and overall phylogenetic characteristics of existing broodstocks. For genetic identification of broodstock species or their genetic purity, analysis of mitochondrial DNA markers is a very useful tool. Moreover, mitochondrial DNA is extensively used for evolutionary and population studies due to its maternal inheritance and fast evolutionary rate.

Currently, many studies apply genetic screening methods to analyze phylogenetic relationships between wild fish species populations or their farmed broodstocks (Sonstebo et al. 2007; Vaha et al. 2007; Maric et al. 2011; Yang et al. 2012). In the case of established fish broodstocks, genetic monitoring

protocols are generally limited to the analysis of several basic parameters of genetic diversity, such as: the number of alleles, allelic richness (A_r), the exact test for the Hardy–Weinberg equilibrium (HWE), the bottleneck test, the inbreeding coefficient (*Fis*), observed (*Ho*) and expected heterozygosities (*He*), effective population size (*Ne*), polymorphism information content (PIC), Shannon–Wiener index (*H'*), Garza–Williamson index (*M*) and genetic differentiation (*Fst*) (Ditlecadet et al. 2006; Araki et al. 2007; Fopp-Bayat et al. 2010; Kaczmarczyk et al. 2012). The application of phylogenetic relationship analysis together with the genetic cluster recognition approach to genetic monitoring of existing fish broodstocks, instead of considering single local stocks, allows a more comprehensive determination of their genetic structure. Knowledge of the overall genetic structure of farmed broodstocks of endangered fish species is very important in sustainable management, enabling the selection of the most suitable stocking material for supplementation of wild populations derived from relevant stocks and characterized by a high level of genetic diversity (Vrijenhoek 1998; Frankham et al. 2010).

At present, all the examined European huchen broodstocks exist as separate and isolated groups propagated only by breeding spawners and maintained solely through internal recruitment. All hatcheries produce roughly from 400 000 to 1 000 000 fry annually, which are used for fishery supplementation in local streams and rivers. Total counts in each of them vary from a few hundred to 800 000 specimens. They are considered as three subgroups: (1) older spawners (10–15 years old), (2) younger spawners (up to 10 years old) and (3) successors (up to 2 years old fish selected for broodstock recruitment). At present, all broodstocks surveyed are viable and not at risk of extinction, however, the broodstock from Slovakia has recently experienced a significant decline in numbers due to a random incident involving a technical malfunction and requires restoration (personal information from hatchery managers).

The applied assignment tests from the ONCOR software package indicate clear homogeneity in the German and Slovakian broodstocks, with the highest probabilities of correct assignment. These parameters were significantly lower in the other broodstocks from Poland and Ukraine included in the study. All the obtained values of correct assignments were above 50%, indicating a significant overall genetic differentiation in the European huchen broodstocks under current study. Each of the approaches applied to genetic analysis grouped these broodstocks into one common genetic clade, proving that they have the most similar genetic structure. Furthermore, most of the methods of genetic analysis showed that the German and Slovakian broodstocks are clearly different from the Polish and Ukrainian ones.

This suggests that the broodstocks under study do not share a common gene pool and are characterized by specific genetic suits, reflecting their adaptation to different environmental conditions in their respective geographical regions. Furthermore, the apparent differentiation of the Slovakian broodstock into two independent subclades, observed during the DAS analysis, may be a consequence of the recent supplementation or establishment of the mentioned broodstock by genetically distant spawners from another stock or wild populations. Noteworthy is the fact that individual multilocus genotype analyses showed the presence of only two genetic clades, suggesting their lower genetic screening abilities compared to other applied genetic cluster recognition methods. According to the present results, the Polish and Ukrainian broodstocks of the European huchen can be used for their complementary supplementation should such a need arise in the future. Moreover, the best available source material for supplementation of the Slovakian broodstock appears to be the German broodstock, but the observed indications of genetic differentiation between them suggest that the first future attempts at supplementing or restoring these broodstocks should be based on locally available individuals from stocked streams or rivers.

It is believed that in small and isolated fish stocks, the risk of significant changes in their genetic structure is much higher due to genetic drift rather than inbreeding (Vrijenhoek 1998; Frankham et al. 2010). Analysis of effective population size (*Ne*) parameters in farmed fish stocks is a very effective tool in determining their susceptibility to genetic drift effects, which is important for proper conservation management planning for endangered fish species (Tringali et al. 1998; Hoarou et al. 2005). It is estimated that *Ne* values greater than 50 are sufficient for short-term prevention of inbreeding and genetic drift events in farmed fish stocks. However, for long-term preservation of good genetic status of reared fish stocks, the values of *Ne* should be at least greater than 100 (Frankham et al. 2010). The estimated effective population sizes (*Ne*) for each studied broodstock were below the threshold value of 50, indicating that they are very susceptible to the impact of genetic drift events accelerating genetic diversity loss and inbreeding effects. Therefore, future management actions should take this precaution into account and seek to improve and develop new effective mating

schemes, such as a more optimal ratio of females to males (1:1), effective breeding procedures that allow equal reproduction pairing of all spawners, reduced impact of inbreeding by changing the recruitment strategy of successors and keeping a high and stable census of spawners. In fact, some traditional and convenient hatchery management procedures are still in place, i.e. the use of a reduced number of males relative to females during controlled reproduction, internal recruitment of successors to maintain a broodstock, and reduced total broodstock census. The major issue concerning all studied broodstocks appears to be a reasonable strategy for obtaining new spawners, which should be based on locally available wild fish. Furthermore, particular care should be taken with the Slovakian broodstock, where the estimated effective population size (*Ne*) was extremely low (*Ne* = 7). The clear subgrouping within the Slovakian broodstock together with the low effective population size may suggest that the current breeding schemes reflect unequal reproduction pairing of spawners.

To conclude, the protocol presented in this study, describing the step-by-step procedure for genetic relationship analysis involving four European huchen broodstocks from Central and Eastern Europe, can be applied as a helpful tool in the management of farmed broodstocks of other endangered salmonid fish species. The applied approach to microsatellite DNA analysis, along with the mathematical genetic data processing method, yielded robust results that can be useful in addressing crucial issues related to the management of farmed fish broodstocks. In addition, the proposed genetics-based management approach appears to be particularly suitable not only for the conservation of the European huchen, but also other rare and valuable salmonid fish species. At the end, it should be stressed that most of the current conservation programs for the European huchen in Europe do not include genetic data in their long-term strategies, where only selected broodstocks are genetically screened, and there are no comparable and comprehensive genetic data on wild populations of the species. Therefore, the results of most of the implemented fishery supplementation measures across Europe are completely unknown in terms of impact on exiting wild populations and effectiveness of stocking strategies. In the future, this issue should receive attention and a new integrated large-scale conservation management strategy based on genetic data should be developed, which would include all existing broodstocks and wild populations of the species.

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