FISHERIES & AQUATIC LIFE (2025) 33: 153 - 165
DOI 10.2478/aopf-2025-0014

RESEARCH ARTICLE

New multiplex PCR assays for estimating genetic diversity in European grayling (*Thymallus thymallus*) by polymorphism of microsatellite DNA

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Received – 8 November 2024/Accepted – 4 November 2025. Published online: 30 September 2025; ©National Inland Fisheries Research Institute in Olsztyn, Poland
Citation: Kaczmarczyk, D., Nitkiewicz, A., Gadomska M. (2025). New multiplex PCR assays for estimating genetic diversity in European grayling (*Thymallus thymallus*) by polymorphism of microsatellite DNA. Fisheries & Aquatic Life 33, 153-165.

Abstract. The European grayling (*Thymallus thymallus* L.) is a vulnerable freshwater fish that plays an important role in the conservation of the biodiversity of European ichthyofauna and is a key species in recreational fishing. Multiplex PCR is a technique used to estimate genetic diversity that saves both time and money. This paper presents three multiplex PCR assays for amplifying 11 microsatellite markers from European grayling DNA. The steps necessary for constructing multiplex assays and verifying genotyping results are described. The compositions of the PCR mixture and the characteristics of the PCR thermal profile recommended for amplification in multiplex mode, and protocols used in genotyping them by using automatic DNA sequences are also described. Consequently, our method enables a near four-fold reduction in time and costs of the PCR stage of studies on genetic variation. Moreover, the next step of analysis (measuring the length of DNA fragments) can be done faster and easier without decreasing the precision or quality of genotyping results. This method was tested by assessing the genetic variation in a grayling stock kept under aquaculture conditions, and it has proved useful in the management of the genetic variation of this species.

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Keywords: conservation genetics, improved molecular tool, technical note

Introduction

The European grayling (*Thymallus thymallus*) is a freshwater species that typically inhabits rivers with cold, clear, well-oxygenated waters (Mallet et al. 2000). This species is very valuable for the conservation of the biodiversity of European rivers and one of the most important fish species for recreational fishing (Lyach and Remr 2019). Unfortunately, during last few decades, grayling populations have declined mostly as a consequence of water pollution, habitat degradation, overfishing, and climate change (Marsh et al. 2021, Mruk 2024). In many countries the European grayling is classified as vulnerable, endangered, or human dependent (Persat 1996, Akimov 2009, Helcom 2013, Ford 2024). The grayling population in Poland requires constant conservation, including stocking with material cultivated in aquaculture (Rich et al. 2018). The conservation of this species can be problematic because populations show high levels of adaptation to specific local environments, which means that even populations inhabiting the

same river can differ significantly (Brzuzan and Jurczyk 2003). Consequently, the translocation of stocking materials among rivers is harmful and can decrease the fitness of conserved populations (Koskinen et al. 2002, Susnik et al. 2004, Duftner et al. 2005). Accordingly, studies on genetic variation are extremely important for preserving the genetic diversity of species and can assist in preventing contamination of native populations with foreign fishes. Consequently, there is a need to establish a highly efficient molecular tool based on multiplex PCR reactions that can be used in the conservation of this species.

The grayling genome is not well studied (Weiss et al. 2021). There is a group of microsatellite markers that has been developed from the DNA of graylings or other Salmonidae species (Olsen et al. 1998, Sušnik et al. 1999, Stamford and Taylor 2005, Junge et al. 2010) that is useful for assessing the genetic variation. (Shrimpton and Clarke 2012). These markers have been used to examine the genetic diversity of the species, but mostly with singleplex PCR (Marić et al. 2011, Shrimpton and Clarke 2012). To date, the number of grayling microsatellite loci that have been successfully matched in sets, enabling their amplification by multiplex PCR is limited (Koskinen and Primmer 2001, Swatdipong 2009, Haddeland et al. 2016).

As with any other fish species, maintaining the genetic variation of grayling populations is key for the survival of the species. A decrease in variation reduces the potential of populations to adapt and renders them vulnerable to changes in environmental conditions and pathogenic infections that can threaten their existence (Pastor et al. 2004, Zemanová et al. 2015). Therefore, maintaining genetic variation and preserving the ability of conserved populations to adapt to environmental conditions should be the basis of conservation and restoration work.

In studies that estimate genetic variation, amplifying DNA fragments using the PCR technique is usually the most costly, time-consuming part of the research. Multiplex PCR is a molecular technique that is intended for the simultaneous amplification of two or more loci in a single PCR reaction (Edwards and Gibbs 1994). Assembling sets of primers into multiplex PCR sets permits amplifying DNA fragments located at multiple loci using only a fraction of the chemicals and time required compared to amplifying them separately. Therefore, the multiplex PCR technique is a much faster, less expensive molecular method that also maintains accuracy. As a result, multiplex PCR is frequently used in population studies based on the analysis of numerous markers, such as microsatellite DNA (Lerceteau-Köhler and Weiss 2006, Rai et al. 2009).

Microsatellite DNA fragments are used commonly as markers of genetic diversity in protected populations (Nielsen and Sage 2001, Kim et al. 2004, Jurczyk 2006, Zemanová et al. 2015), to monitor the genetic fitness of conserved populations, and to track their interactions with other populations (Wąs and Wenne 2002). The degree of polymorphism of microsatellite loci (the number of repeat DNA motifs) is a measure of the level of genetic variability in a stock or natural population (Guichoux et al. 2011). In such studies, the advantages of the multiplex PCR technique are evident, because it consumes less reagents and reduces the time required to amplify microsatellite DNA fragments.

Accordingly, the aim of this study was to find groups of primer pairs from the singleplex studies mentioned above that could be successfully used together in one PCR reaction. Additional aims were to optimize the reaction environment and the thermal profile of the reaction to make it a more useful tool for estimating the genetic variability of this species as well as testing this method on stocks that are used to support local populations of this species.

Material and methods

Sampling and preservation of samples

The material (fin fragments) was collected from 30 grayling spawners (15 females and 15 males) from the broodstock of the Department of Salmonid

Research in Rutki (National Inland Fisheries Research Institute), Poland, in the spring of 2024. Fin fragments (7 x 7 mm) were collected from the spawners and preserved by drying. Before sampling, the fish were marked with Biomark HPT8 microchip tags (Biomark, USA) to enable their identification.

DNA extraction

A Sherlock AX DNA extraction and purification kit (A&A Biotechnology, Gdańsk, Poland) was used to extract DNA from the fin samples, in accordance with the protocol provided by the manufacturer. The degree of DNA fragmentation was evaluated by electrophoresis in 1.5% agarose gel, and the amount of DNA was measured with spectrophotometry at a wavelength of 260 nm. Samples that did not show traces of fragmentation and contained at least 80 pg μL^{-1} DNA were used to amplify microsatellite loci in the next stage of the experiment. If a DNA sample failed to meet the above criteria, the extraction procedure was repeated.

Choice of primers and optimization of PCR reaction conditions

Sequences of primers used to amplify microsatellites of grayling were obtained from papers by Sušnik et al. (1999) and Junge et al. (2010). Eleven sets of primer pairs were selected which, according to the aforementioned studies, would enable amplification of a microsatellite with considerably high polymorphism (Table 1), and had a similar annealing temperature (range 55–60°C). The initial tests involved singleplex PCR by using unlabelled primer sets. The initial PCR mixture composition was based on proportions given in the papers. During our work, the number of primers, the DNA template, and the proportions of other PCR components were adjusted to meet the recommendations given by the manufactures of the chemicals used in our laboratory. Reaction mixtures were prepared in a total volume of 25 µl with a 40 ng DNA template, a 1x PCR reaction buffer (50 mM KCl, pH 8.5; Triton X-100), 0.4 mM of each primer, 0.25 mM of each deoxynucleotide triphosphate (dNTP), 3.3 mM $MgCl_2$, and 0.6 unit Go Taq flexi DNA polymerase (Promega, Madison, WI, USA). Re-distilled water (Ambion, USA) was used to bring the reaction mixture to the desired final volume. To identify and determine the optimal annealing temperature for all microsatellites, the PCR reaction was conducted under temperature gradient conditions in a MASTERCYCLER X50a thermocycler (Eppendorf, Germany). At this stage the thermal profile of the PCR reaction was set to mimic conditions reported in papers published 15–25 years ago with some modifications (e.g. slightly higher upper range of annealing temperatures = 62°C), which resulted from the different chemicals used in our laboratory and the recommended protocols for their use. The following test parameters were selected: initial denaturation at 95°C for 15 min, followed by 38 amplification cycles (94°C, 30 s; gradient from 56–62°C: 1:30 min; 72°C, 1 min), and final elongation at 60°C for 30 min. The PCR product was verified by electrophoresis in a 2% agarose gel and stained using ethidium bromide (Invitrogen, USA).

To select the optimal working conditions for the primers, different variants of the reaction mixture were tested at temperatures ranging from 48 to 70°C. DNA from eight randomly selected samples was used as the matrix in the tests. Pairs of primers that permitted using a clear PCR product with a length close to that obtained in the findings of previous research (Sušnik et al. 1999, Junge et al. 2010) were synthesized again as oligonucleotides by Applied Biosystems (USA); the forward primers were marked with phosphamide dyes 6FAM, VIC, NED, PET.

Construction of primer sets for the multiplex PCR assay

To primer sets, the primers considered were those that had a common amplification temperature, as determined during their amplification in singleplex mode (Table 1). The primers were assigned into multiplex sets following Roche's guidelines, as

Table 1
Information about microsatellite loci and primer pairs used for amplification (Sušnik et al. 1999, Junge et al. 2010)

Locus	Genebank assesion nr	Primer sequence	Repeat motiff	Product size (bp)	Annealing temperature (°C)
BFRO005	AF115407	F: CGCATCTGTATGAAAAACCT	$(CA)_{17}$	111-134	55
		R: TGGTTTGGTAGGAGTTTCGT			
BFRO006	AF115408	F: GCCTGGTTTTACCCTTTAGA	$(CA)_{16}$	134-140	55
		R: AGGCATTTTACACTGGCATT			
BFRO007	AF115409	F: AGACCCCCAAAAACTATGCT	$(AG)_{13}$	176-182	55
		R: TAAGGTCCCCAACACTACGA			
BFRO008	AF128890	F: TCTCCCCACTGTAAGTACGC	$(GA)_{26}$, $(CA)_{24}$	223-237	55
		R: GCATTGATTGTCCTACATTA			
BFRO009	AF128891	F: AAATTGTCCCCGTTGGCAGA	$(CA)_8$, $(AC)_4$, $(AC)_8$, $(CA)_6$	239-241	55
		R: ACATACACCGCAACACCCAG			
Tth-214	GU225712	F: TGGTGCCAGTTAGATAGTCA	$(CAA)_{15}$	292-313	58
		R: GCTTCACATTTATCCTATGATT			
Tth-313	GU225716	F: AAACCAGTCCAAGCGAGAG	$(GAGT)_{22}$	169-201	60
		R: CTCCTGTTTATCACATGA			
Tth-414	GU225719	F: GTCGGGACATGGACTCTACA	$(AGAT)_{21}$	341-409	60
		R: GCAATGCCTCTTTATAGCTT			
Tth-415	GU225720	F: GGGATCAATAAAGTTCTATC	$(ATCT)_{18}$	176-434	59
		R: GCTATTAAACATTAAGGGT			
Tth-433	GU225723	F: AATGATGTCAATTAGCCTAT	$(AGAT)_{35}$	159-163	59
		R: GTTTACAGACTTAGTGAA			
Tth-445	GU225725	F: TGACGGCTACAGGAATTGT	$(GATA)_{20}$	375-497	59
		R: CCACAGAGGGTTCTACATTG			

recommended in construction sets for multiplex PCR. Of these, primer pairs that had different dyes were checked for complementarity with the Primer Pooler v1.88 (Brown et al. 2017) (http://ssb22.user.srcf.net/pooler/). Those with a strong tendency to create primer-dimer structures were excluded. This complementarity was expressed as values of ΔG^{o}_{37} (Watson-Crick nearest-neighbor thermodynamic parameter for temperature of 37°C) (He et al. 1991). Those with high complementarity (a low value of ΔG^{o}_{37}) were replaced by fragments with lower complementarity (a higher value of ΔG^{o}_{37}). The lowest acceptable value of ΔG^{o}_{37} within a set of primers for the multiplex PCR reaction was -9.

Multiplex PCR amplification was performed with a QIAGEN Multiplex PCR Kit. Following the manufacturer's protocol, the PCR primers were suspended in TE buffer to reach a base concentration of 100 pmol/µl. The final concentration of each pair of the PCR primers in the multiplex PCR set was adjusted to reach a balanced yield of PCR products. The composition of the PCR mixture followed the manufacturer's recommendations and contained: 7.5 µl of QIAGEN Multiplex PCR mastermix, 3 µl of Q solution, (0.56–1.82 µmol of each primers) (Table 2), and deionized water to reach a final volume of 15 µl. The optimization of the multiplex PCR conditions included adjusting the thermal profile of multiplex PCR. The starting point of these modifications was based on the Universal Multiplex Cycling Protocol (QIAGEN), which was modified to meet the requirements of each multiplex PCR set. It contained initial

Table 2
Optimal 5' labeling, number of primers in the 15 µl mixture, annealing temperature, allele range, number of repeats, number of identified alleles (n) observed (*Ho*) and expected (*He*) heterozygosity, (*m*) value of the Garza-Williamson Index

Set	Locus	5' labeling	Primers each (µmol)	Annealing temperature (°C)	Allele range (bp)	Repeat numbers	*n*	*Ho*	*He*	*m*
Multiplex I	*BFRO005*	6FAM	0.56	56	111-125	4-11	4	0.50	0.47	0.50
	BFRO006	PET	1.12		143-145	40-41	2	0.42	0.39	1.00
	BFRO007	VIC	0.70		184-192	14-18	2	0.44	0.43	0.40
	BFRO008	6FAM	1.82		205-219	45-52	2	0.28	0.35	0.25
	BFRO009	NED	0.70		245	65	1	0.00	0.00	-
Multiplex II	*Tth-214*	PET	1.54	58	294-315	11-18	5	0.78	0.74	0.63
	Tth-313	VIC	1.68		115-167	8-21	3	0.36	0.47	0.21
	Tth-414	VIC	1.12		340-348	5-7	2	0.64	0.51	0.67
	Tth-445	NED	0.84		353-393	15-25	4	ž0.64	0.62	0.36
Multiplex III	*Tth-433*	6FAM	1.82	56	160-168	1-3	2	0.19	0.18	0.60
	Tth-415	6FAM	1.82		272-288	34-38	3	0.31ž	0.44	0.67

denaturation (95°C for 15 min), followed by denaturation (94°C, for 90 s), annealing at a temperature specific for each set (Table 2) for 90 s, extension at 72°C for 90 s, and a final extension at 72°C for 10 min. The PCR reaction consisted of 35 cycles including the denaturation, annealing, and extension steps. To compare the quality of multiplex PCR results with singleplex results, the same set of chemicals and PCR protocol were used. The optimization of multiplex PCR started from the selection of two microsatellite loci that required similar amplification conditions and were applied to them together. The results of amplification yield and the presence of additional unspecific peaks were compared with singleplex amplification. If PCR products of the same length were produced, the primer pairs were considered suitable for joint amplification. Another pair was then tested with these pairs and, if found suitable, it was added to the set. In this way, three sets of primer pairs were created and named Multiplex I, II, and III.

To optimize the performance of the multiplex PCR reaction, the conditions for amplifying individual loci within the multiplexes were optimized in accordance with the guidelines developed by Henegariu et al. (1997) and recommendations provided by Fishback et al. (1999). The proportions of primers within each multiplex assay were optimized individually to provide similar amounts of amplicons of each of the microsatellite fragments.

Multiplex PCR amplification of microsatellites was performed on 96 well plates, which permitted the direct transfer of PCR products to the plate used for fragment analysis and genotyping. This eliminated the need to transfer the PCR product from several plates to the plate used for length measurements, which was done with an automated capillary DNA sequencer, which made preparation to this stage of analysis much faster and decreased the likelihood of laboratory error.

Fragment analysis and genotyping

Automatic capillary electrophoresis was used to verify the usefulness of individual microsatellite fragments, construct multiplex PCR assays (Butler et al. 2001). The length of the DNA fragment was measured by using an Applied Biosystems 3130 Genetic Analyser DNA sequencer. Determining the size of DNA fragments was performed against the GeneScan 500LIZ Size- Standard (Applied Biosystems) in multiplex mode. This enabled measuring fragments simultaneously with attached phosphamide dyes (6FAM, VIC, NED, PET). The

mixture used for genotyping included: 19 μl highly deionized formamide, 0.5 μl GeneScan LIZ500 Size Standard, and 0.5 μl multiplex PCR product. The separation of the DNA fragments was performed in 36 cm capillary arrays and POP-7 polymer. Genotyping was performed with GeneMapper 3.0 (Applied Biosystems).

Verification the results of amplifications performed using multiplex PCR sets

Eight random samples of grayling DNA from the Department of Salmonid Research in Rutki were used to verify the multiplex PCR protocols. This comparison was performed in two stages. First, the results of multiplex and singleplex amplification protocols of microsatellites from the same sample were compared using horizontal agarose gel electrophoresis. At this stage, PCR product yield and the presence of additional nonspecific bands was assessed. When PCR product yield obtained with multiplex and singleplex reactions was similar and there were no visible additional nonspecific bands, the samples were qualified for the next stage of verification in an automatic DNA sequencer. At this stage, PCR product readability was assessed and compared between the multiplex and singleplex protocols. Moreover, the presence of additional peaks was inspected. Next, the microsatellite loci were genotyped with GeneMapper 3.0 (Applied Biosystems). If the genotyping results of microsatellites amplified with multiplex and singleplex PCR were the same and no additional peaks were found that disturbed genotyping, this multiplex was qualified as being successful in the amplification of microsatellites included in this set.

Polymorphism and genetic variation

Polymorphism at the loci investigated and genetic diversity of this stock was assessed with indicators such as observed heterozygosity (H_o), expected heterozygosity (H_e), the number of alleles (A_n), and the inbreeding coefficient (F_{IS}). The values of these indicators were calculated with microsatellite analyser (MSA) software (Dieringer and Schlötterer 2003). An exact Hardy Weinberg (H-W) test (Nei 1987) was used to detect deviations from H-W equilibrium. The test was performed separately for each locus in each population as well for all loci in given populations. This test was performed with Arlequin 3.5.2 (Excoffier et al. 2010). The number of steps in the Markov chain was 1,000,000 and the number of dememorization steps was 100,000. Deviations were considered significant at $P \leq 0.05$. MSA software (Dieringer and Schlötterer 2003) was used to assess the reduction of genetic variation resulting from bottleneck or founder effects. The occurrence of bottleneck or founder effects, and their influence on intra-population genetic variability was based on the Garza-Williamson *M* index (the number of alleles divided by the allelic range), which included Excoffier's adjustment, was calculated with Arlequin 3.5.2 (Excoffier et al. 2010).

Results

All 11 loci were assembled in three multiplex assays that were amplified with three multiplex PCR reactions. The results of the amplifications performed with multiplex and singleplex PCR protocols were the same. Optimal conditions of the amplification of Multiplex I, II, and III are presented in Table 2.

Primer complementarity and heterodimer tendency

Most primer pairs in the multiplex assays did not tend to form heterodimers. The average value of ΔG^{o}_{37} (Gibbs Free Energy at a temperature of 37°C) for Multiplex I was -0.09 kcal mol^{-1}; for Multiplex II — -0.13 kcal mol^{-1}; and for Multiplex III — 1,24 kcal mol^{-1}. The highest complementarity in Multiplex I was between primers: *BFRO006R* and *BFRO009F* (ΔG^{o}_{37} = -1.18 kcal mol^{-1}), *BFRO006F* and *BFRO006R* (ΔG^{o}_{37} = -1.08 kcal mol^{-1}), and *BFRO005F* and *BFRO006F* (ΔG^{o}_{37} 1.01 kcal mol^{-1}). In Multiplex II, the highest complementarity was detected between

primers *Tth-414F* and *Tth-313F* (ΔG^{o}_{37} 2.03 kcal mol^{-1}). In Multiplex III, all ΔG^{o}_{37} values were above 0 with the lowest value between the primers *Tth-433F and Tth-415R* ΔG^{o}_{37} (0.26 kcal mol^{-1}). Simulations of the heterodimer tendency in these multiplex sets estimated with the ΔG^{o}_{37} parameter were confirmed by the results of automatic capillary electrophoresis.

After the optimization of the number of primers and the annealing temperature, Multiplex I clearly amplified all five microsatellites with a very few unspecific PCR products. The peaks of the microsatellites were clearly identifiable. The fragments containing microsatellite DNA emitted much stronger signals than unspecific PCR products (Fig. 1), consequently the samples were easy to genotype (Fig. 1A-D).

Similar results of multiplex PCR were observed in Multiplex II by which all four samples were amplified successfully (Fig. 2). Amplified fragments containing microsatellite DNA were much stronger than unspecific products and easy to genotype (Fig. 2A-C).

Multiplex III, which contained two microsatellites that were both marked with the same 5' labelling (6FAM), produced a significant amount of unspecific products (Fig. 3). The strength of the signal emitted by these unspecific products was much lower than the target products containing microsatellites *Tht-433* and *Tht-415*, and the unspecific products were not problematic in the genotyping of these loci.

Genetic variation in the Rutki stock and its usefulness in grayling conservation

In the stock investigated, almost all the microsatellites were polymorphic. Only one locus (*BFRO009*) was monomorphic; therefore, some indicators of genetic variations, such as the *m* value of Garza-Williamson index and deviations from the Hardy-Weinberg Equilibrium, were not calculated. The polymorphism of other loci was not high ranging from two to five alleles. The most polymorphic was locus *Tth-214*, and the other five loci had only two alleles. Overall, 30 alleles were found across 11 loci investigated loci. The average number of alleles/loci was low A_n = 2.73. The average observed heterozygosity (H_o) calculated across all loci was not high (H_o = 0.42) and was close to the average expected heterozygosity (H_e = 0.40). These values indicated a reduction of genetic variation as a result of the domestication of this stock. There were no significant deviations from the Hardy-Weinberg Equilibrium in any of the loci investigated (P > 0.05). The lack of deviations from the Hardy-Weinberg Equilibrium indicated that there were no factors that could have significantly affected the genetic variation in this stock. The mean *m* value of the Garza-Williamson index was 0.53, indicating that the genetic variation in this broodstock was probably reduced due to founder or bottleneck effects (Table 2). Any impact of inbreeding or outbreeding on the genetic variation of this stock was very limited. The inbreeding coefficient was F_{IS} = 0.01 and close to a neutral value (F_{IS} = 0). Summarizing the values of genetic variation indicators, the fish from Department of Salmonid Research in Rutki were typical of fish living in a small population that is strictly adapted to its local environment. The genetic variation of the stock in Rutki was probably decreased as a result of the founder effect. On the other hand, this stock is not inbred, therefore it is a valuable source of stocking material, especially for supporting the source population of grayling. To prevent domestication, which can decrease the survivability of fish in natural conditions, importing material from native populations is recommended.

Discussion

This paper presents three new multiplex PCR sets for effectively amplifying grayling microsatellites. With these primer pairs, 11 microsatellites that were amplified separately by Sušnik et al. (1999) and Junge et al. (2010) by using singleplex protocols, can be amplified in three multiplex PCR reactions. Although 11 microsatellite loci combined into three multiplex PCR assays are considered sufficient for estimating genetic

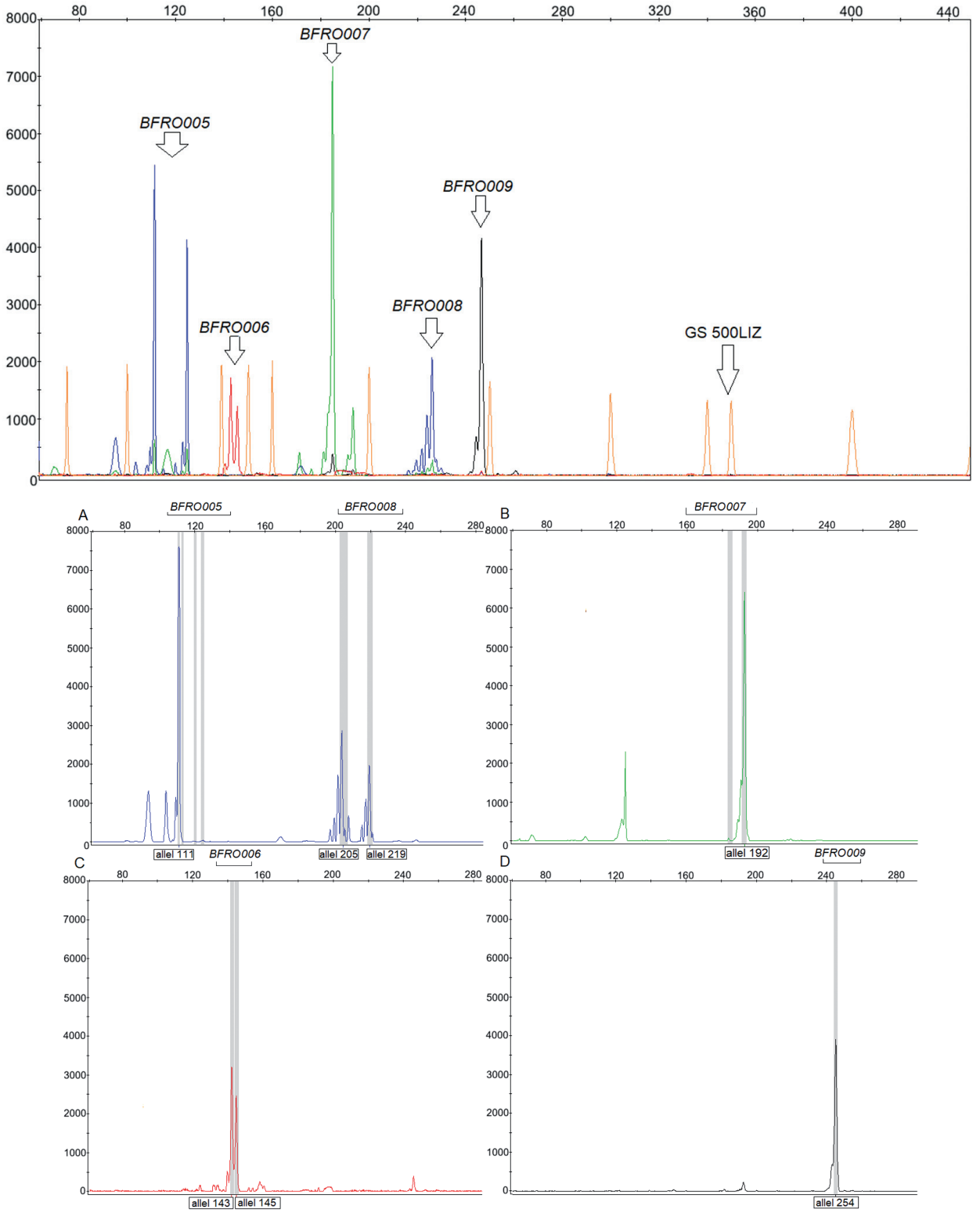

Figure 1. Electropherogram DNA containing microsatellites from Multiplex I. Orange line – GeneScan 500LIZ Size Standard; blue line – microsatellites *BFRO005* and *BFRO008*; red line – microsatellite *BFRO006*; green line – microsatellite *BFRO006*; black line – microsatellite *BFRO009*. A – *BRFO005* (alleles: 111bp, 205bp) and *BFRO008* (alleles: 205bp, 219bp); B – *BFRO007* (alleles: 192bp, 192bp); C – *BFRO006* (alleles: 143bp, 145bp); D – *BFRO009* (alleles:154bp, 154bp). Grey fields – ranges of PCR product length (bins) used by GeneMapper 3.0 for determining alleles.

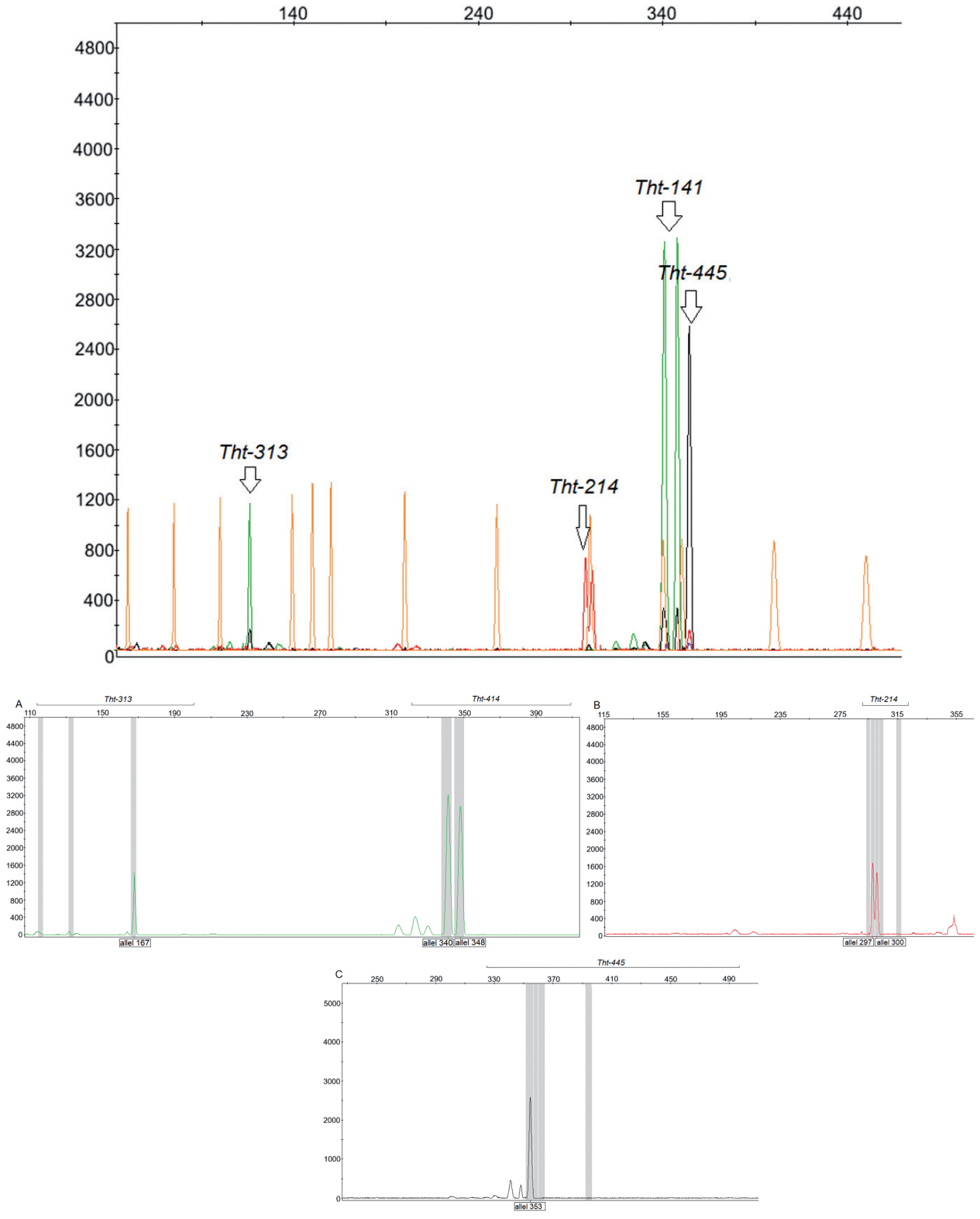

Figure 2. Electropherogram DNA containing microsatellites from Multiplex II. Green line – microsatellites *Tht-313* and *Tht-414*; red line – microsatellite *Tht-214*; black line – *Tht-445*. A – *Tht-313* (alleles: 167bp, 167bp) and *Tht-414* (alleles: 340bp, 348bp), B. *Tht-214* (alleles: 297bp, 300bp); C – *Tht-445* (alleles: 353bp, 353bp); grey fields – ranges of PCR product length (bins) used by GeneMapper 3.0 for determining alleles.

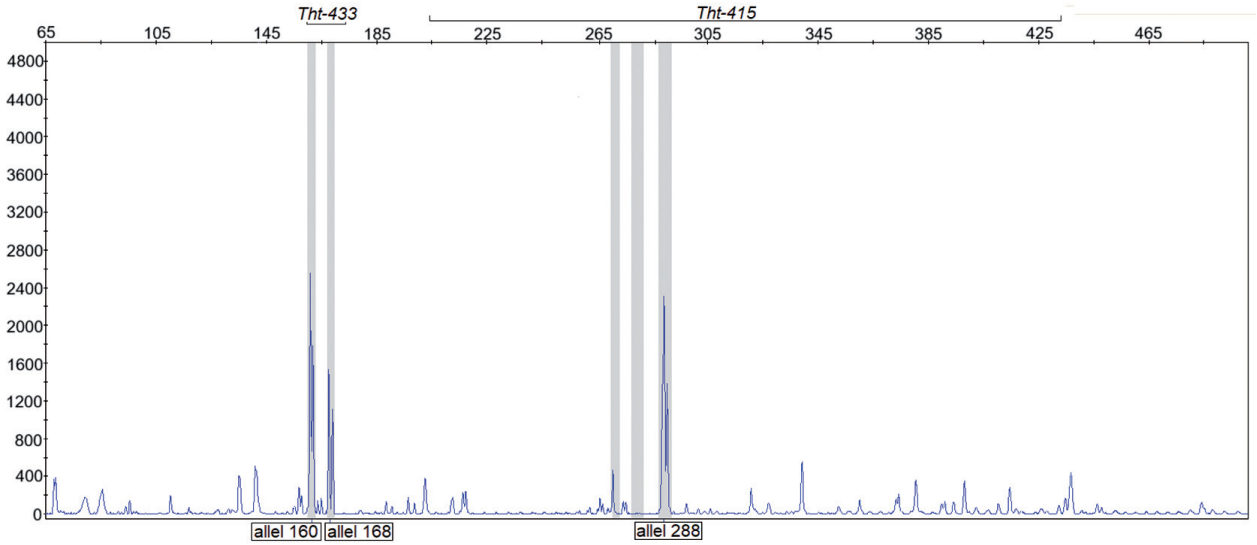

Figure 3. Example of genotyping the microsatellites from Multiplex III. Locus – *Tht-433* (alleles: 160bp, 168bp); locus – *Tht-415* (alleles: 288bp, 288bp); grey fields – ranges of PCR product length (bins) used by GeneMapper 3.0 for determining alleles.

variability, if an even better estimate is desired, our primers can be used in conjunction with those previously developed by Olsen et al. (1998), Stamford and Taylor (2005), and Junge et al. (2010). Future work in this area could focus on finding additional sets of previously published primer pairs and adding them to the Multiplex III assay, because it only contains two pairs of primers. The efficacy of the application of this method in studies on genetic variation in other species of grayling or salmonids is not clear. Some of the markers, including multiplex sets, have proved to be useful in studies on species of other European grayling (Junge et al. 2010). This indicates that the three proposed Multiplex PCR sets may be useful in studies of the genetic variation of species other than European grayling, but this remains to be tested.

Our method enables nearly four-fold savings of time, expensive reagents, and DNA compared to the singleplex amplification of the same loci, without sacrificing the quality of analysis. Because of the low compatibility among primers in each set, these primer pairs effectively amplify the target loci. Moreover, by eliminating the need of pipetting PCR product amplified during several singleplex reactions to plates for length fragment analysis by automatic DNA sequencer, time is saved and the likelihood of laboratory error at this stage of analysis is reduced. Thus, it increases the reliability of determining the genetic variation of European grayling.

The multiplex sets described in this paper are an alternative to or supplement for existing sets used in multiplex PCR (Koskinen and Primmer 2001, Swatdipong 2009, Haddeland et al. 2016) in studies on the genetic variation of European grayling. The effectiveness of these sets for estimating genetic variability was demonstrated with the broodstock in Rutki. These sets of primers proved to be useful for estimating genetic variation in stocks of grayling and can be used in the conservation of this species.

The results of our studies can be used in various tasks in grayling conservation:

- estimating genetic variation in populations of grayling for evaluating their genetic diversity and tracking eventual crossbreeding with stocking material that have different genetic characteristics;
- assessing the success of the conservation of this species and the survivability of material produced in aquaculture;
- choosing an optimal strategy for the conservation of grayling, such as identifying populations that are most similar to conserved or

extinct populations and determining optimal locations for fish stocking;
- combined with microchip tagging, this method permits identifying the genetic characteristics of spawners used as a source of material for conservation and will allow them to be matched in pairs according to their genetic differences (Kaczmarczyk and Wolnicki 2020);
- managing genetic variation in banks of cryopreserved gametes and identifying the optimal set that can potentially transfer the most genetic variation to the next generation (Kaczmarczyk and Wolnicki 2022);
- combining multiplex PCR techniques, the genetic profiles of spawners, and gamete cryopreservation will permit managing genetic diversity in broodstocks used for producing stocking material, and for maintaining grayling populations that are dependent on humans while also safeguarding genetic variations of grayling populations in cryopreserved sperm banks.

By using the multiplex PCR sets developed and tested in this research, the implementation of all the tasks discussed above will be much faster and less expensive, while maintaining the accuracy of the results.

Summary

This paper describes a new method for assessing genetic variation in European grayling based on three PCR multiplex sets. This method includes 11 microsatellite markers that can be amplified using only three PCR reactions, saving time and the chemicals required for this kind of analysis. The method described in this paper was tested on a European grayling stock at the Department of Salmonid Research in Rutki and proved to be successful for assessing the genetic variation of this species. Consequently, this method is a fast, cost-efficient tool for screening genetic variation in grayling in both Polish and European rivers. It can be included in conservation programs of European grayling in Poland and Europe and is a useful tool for breeding this species in aquaculture.

Acknowledgments. This study was supported by the Agriculture Restructurisation and Modernisation Agency (ARIMR) within project No. 00061-6521.5-OR1400-0001/20/21 in 202–2026, and by research topic Z-020 of the Department Pond Fishery, National Inland Fisheries Research Institute, Olsztyn, Poland. The authors are grateful to Stefan Dobosz, Department of Salmonid Research in Rutki, National Inland Fisheries Institute in Olsztyn, Poland for providing material for this study.

Authors contributions: Conceptualization, D.K.; Methodology, A.N and M.G., Validation, D.K.; Formal Analysis, D.K.; Investigation, M.G., D.K. and A.N.; Resources, D.K. .; Data Curation, D.K.; Writing – Original Draft Preparation, D.K., A.N.; Writing – Review & Editing, D.K., A.N.; Visualization, D.K.; Supervision, D.K.; Project Administration, D.K.; Funding Acquisition, D.K.

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References

Akimov, I. 2009. The Red Data Book of Ukraine. Animals. Global Consulting, Kyiv, 1-486.

Brown, S.S., Chen, Y-W., Wang, M., Clipson, A., Ochoa, E., Du, M-Q. (2017). PrimerPooler: automated primer pooling to prepare library for targeted sequencing. Biology Methods and Protocols. Oxford University Press. 2(1).

Brzuzan, P., Jurczyk, Ł. (2003). Analysis of mtDNA sequences of European grayling, *Thymallus thymallus*, from southwestern Poland. Archives of Polish Fisheries, 11, 301-306.

Butler, J.M., Ruitberg, C.M., Vallone, P.M. (2001). Capillary electrophoresis as a tool for optimization of multiplex PCR reactions. Fresenius Journal of Analytical Chemistry, 369(3), 200-205.

Dieringer, D., Schlötterer, C. (2003). Microsatellite analyser (MSA): a platform independent analysis tool for large microsatellite data sets. Molecular Ecology Notes, 3(1), 167-169.

Duftner, N., Koblmüller, S., Weiss, S., Medgyesy, N., Sturmbauer, C. (2005). The impact of stocking on the genetic structure of European grayling *Thymallus*

thymallus, Salmonidae) in two alpine rivers. Hydrobiologia, 542(1), 121-129.

Edwards, M.C., Gibbs, R.A. (1994). Multiplex PCR: advantages, development, and applications. PCR Methods and Applications, 3(4), S65-S75.

Excoffier, L., Lischer, H.E. (2010). Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Molecular Ecology Resources, 10(3), 564-567.

Fishback, A.G., Danzmann, R.G., Sakamoto, T., Ferguson, M.M. (1999). Optimization of semi-automated microsatellite multiplex polymerase chain reaction systems for rainbow trout (*Oncorhynchus mykiss*). Aquaculture, 172(3-4), 247-254.

Ford, M. (2024). *Thymallus thymallus*. The IUCN Red List of Threatened Species 2024: e.T266178365A135093349. https://dx.doi.org/10.2305/IUCN.UK.2024-2.RLTS.T266178365A135093349.en.

Garza, J.C., Williamson, E.G. (2001). Detection of reduction in population size using data from microsatellite loci. Molecular Ecology,10(2), 305-318.

Haddeland, P.J., Junge, C., Serbezov, D., Vøllestad, L.A. (2015). Genetic parentage analysis confirms a polygynandrous breeding system in the European grayling (*Thymallus thymallus*). PloS one, 10(3), e0122032.

He, L., Kierzek, R., SantaLucia, Jr J., Walter, A.E., Turner, D.H. (1991). Nearest-neighbor parameters for G.U mismatches. Biochemistry, 30, 11124-11132.

HELCOM, (2013). HELCOM Red List of Baltic Sea species in danger of becoming extinct. Balt. Sea Environment Proceedings, 140, 1-72.

Henegariu, O., Heerema, N.A., Dlouhy, S.R., Vance, G.H., Vogt, P.H. (1997). Multiplex PCR: critical parameters and step-by-step protocol. Biotechniques, 23(3), 504-511.

Junge, C., Primmer, C.R., Vøllestad, L.A., Leder, E.H. (2010). Isolation and characterization of 19 new microsatellites for European grayling, *Thymallus thymallus* (Linnaeus, 1758), and their cross-amplification in four other salmonid species. Conservation Genetics Resources, 2(Suppl 1), 219-223.

Jurczyk, Ł. (2006). The genetic characteristics of selected grayling populations (*Thymallus thymallus* Linnaeus, 1758) based on the molecular markers. PhD thesis, University of Warmia and Mazury (in Polish).

Kaczmarczyk, D., Wolnicki, J. (2020). Genassemblage 2.0 software facilitates conservation of genetic variation of captively propagated species. Scientific Reports, 10(1), 17871.

Kaczmarczyk, D., Wolnicki, J. (2022). Management of genetic variation in the gamete bank of the endangered lake minnow *Eupallasella percnurus*, using Genassemblage 2.2 software. Animals, 12(23), 3329.

Kim, J.E., Withler, R.E., Ritland, C., Cheng, K.M. (2004). Genetic variation within and between domesticated chinook salmon, *Oncorhynchus tshawytscha*, strains and their progenitor populations. Environmental Biology of Fishes, 69(1), 371-378

Koskinen, M.T., Primmer, C.R. (2001). High throughput analysis of 17 microsatellite loci in grayling (*Thymallus* spp. Salmonidae). Conservation Genetics, 2(2), 173-177.

Koskinen, M.T., Sundell, P., Piironen, J., Primmer, C.R. (2002). Genetic assessment of spatiotemporal evolutionary relationships and stocking effects in grayling (*Thymallus thymallus*, Salmonidae). Ecology Letters, 5(2), 193-205.

Lerceteau-Köhler, E., Weiss, S. (2006). Development of a multiplex PCR microsatellite assay in brown trout *Salmo trutta*, and its potential application for the genus. Aquaculture, 258, 641-645.

Lyach, R., Remr, J. (2019). The effect of a large-scale fishing restriction on angling harvest: a case study of grayling *Thymallus thymallus* in the Czech Republic. Aquatic Living Resources, 32, 11.

Mallet, J.P., Lamouroux, N., Sagnes, P., Persat, H. (2000). Habitat preferences of European grayling in a medium size stream, the Ain River, France. Journal of Fish Biology, 56(6), 1312-1326.

Marić, S., Razpet, A., Nikolić, V., Simonović, P. (2011). Genetic differentiation of European grayling (*Thymallus thymallus*) populations in Serbia, based on mitochondrial and nuclear DNA analyses. Genetics Selection Evolution, 43(1), 2, http://www.gsejournal.org/content/43/1/2

Marsh, J.E., Cove, R.J., Britton, J.R., Wellard, R.G., House, A., Gregory, S.D. (2021). Medium-term environmental changes influence age-specific survival estimates in a salmonid population. Freshwater Biology, 66(8), 1530-1545.

Mruk, A., Kucheruk, A., Bielikova, O., Čiampor Jr, F. (2024). Current state of the European grayling (*Thymallus thymallus* L.) in Ukraine and conservation strategy for population support. A review. Journal for Nature Conservation, 81, 126676.

Nei, M. (1987). Molecular Evolutionary Genetics. Columbia University Pres, New York, USA.

Nielsen, J.L., Sage G.K. 92001). Microsatellite analyses of the trout of northwest Mexico. Genetica, 111, 269-27.

Olsen, J.B., Bentzen, P., Seeb, J.E. (1998). Characterization of seven microsatellite loci derived from pink salmon. Molecular Ecology, 7, 1087-1089.

Pastor, T., Garza, J. C., Allen, P., Amos, W., Aguilar, A. (2004). Low genetic variability in the highly endangered Mediterranean monk seal. Journal of Heredity, 95(4), 291-300.

Persat, H. (1996). Threatened populations and conservation of the European grayling, *Thymallus thymallus* (L., 1758). In: Conservation of endangered fishes of Europe

(Ed.) Kirchhofer A, Hefti D., Birkhäuser Verlag, Basel: 233-247.

Rai, A. J., Udar, N., Saad, R., Fleisher, M. (2009). A multiplex assay for detecting genetic variations in CYP2C9, VKORC1, and GGCX involved in warfarin metabolism. Clinical Chemistry, 55(4), 823-826.

Rich, J., Cove, R.J., Taylor, R.G. (2018). European grayling conservation, ecology & management: a practical conservation guide for the United Kingdom. Grayling Research Trust, UK.

Shrimpton, J.M., Clarke, A.D. (2012). Genetic analysis of Arctic grayling population structure in the Williston Watershed: samples from the Finlay River. Fish and Wildlife Compensation Program – Peace Region Report No. 354.

Stamford, M.D., Taylor, E.B. (2005). Population subdivision and genetic signatures of demographic changes in Arctic grayling (*Thymallus arcticus*) from an impounded watershed. Canadian Journal of Fisheries and Aquatic Sciences, 62(11), 2548-2559.

Sušnik S., Snoj A., Dovč P. (1999). Microsatellites in grayling (*Thymallus thymallus*): comparison of two geographically remote populations from the Danubian and Adriatic river basin in Slovenia. Molecular Ecology, 8: 1756-1758.

Sušnik, S., Berrebi, P., Dovč, P., Hansen, M.M., Snoj, A. (2004). Genetic introgression between wild and stocked salmonids and the prospects for using molecular markers in population rehabilitation: the case of the Adriatic grayling (*Thymallus thymallus* L. 1785). Heredity, 93(3), 273-282.

Swatdipong, A., Vasemägi, A., Koskinen, M.T., Piironen, J., Primmer, C.R. (2009). Unanticipated population structure of European grayling in its northern distribution: implications for conservation prioritization. Frontiers in Zoology, 6(1), 6, doi:10.1186/1742-9994-6-6

Weiss, S.J., Gonçalves, D.V., Secci-Petretto, G., Englmaier, G.K., Gomes-Dos-Santos, A., Denys, G.P., ... & Froufe, E. (2021). Global systematic diversity, range distributions, conservation and taxonomic assessments of graylings (Teleostei: Salmonidae; *Thymallus* spp.). Organisms Diversity & Evolution, 21(1), 25-42.

Zemanová, B., Hájková, P., Hájek, B., Martínková, N., Mikulíček, P., Zima, J., Bryja, J. (2015). Extremely low genetic variation in endangered Tatra chamois and evidence for hybridization with an introduced Alpine population. Conservation Genetics, 16(3), 729-741.