

Polymorphism of microsatellite loci – a tool in studying biodiversity of paddlefish aquaculture broodstock

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ABSTRACT

American paddlefish (*Polyodon spathula*) is a new species in Polish aquaculture, its broodstocks are few and small, and it is possible that all mature fish originated from only a few spawners. Studies on polymorphism of highly variable microsatellite DNA allow revealing genetic characteristics of individual spawners as well as estimation of genetic variation within and divergence

between broodstocks. This paper describes optimised protocols for isolation of DNA from fin tissues, amplification of nine microsatellite loci using PCR technique, and for fish genotyping using automatic capillary DNA sequencer. Our technique was tested towards the fin samples taken from all paddlefish reared in Poland and approaching their sexual maturity; the study included also samples taken from 47 fish of the Ukrainian breeding centre (Gorny Tykich).

INTRODUCTION

Microsatellite DNA, also known as SSRs (simple sequence repeats) or microsatellites, are arrays of tandem repeats of (usually) 2–6 bp sequences (O'Connell and Wright 1997). They are highly abundant and interspersed throughout the genome. Microsatellites are one of the most polymorphic sequences in eukaryotic genomes. They are inherited according to Mendel laws and they constitute excellent markers for studying genetic variation in wild populations and in domesticated fish stocks (Wright 1993). Highly variable microsatellites are flanked by evolutionary conserved fragments of DNA, known as flanking regions. Studies of polymorphism of microsatellites allow revealing genetic characteristics of individual spawners as well as estimation of genetic variation in broodstocks and are widely applicable in population genetics and in conservation biology.

American paddlefish (*Polyodon spathula*) is a new species in Polish aquaculture. In the USA it is frequently reared in polyculture with channel catfish (Mims 2001). The studies accomplished in Polish, Hungarian and Romanian breeding centers proved that paddlefish performs very well in polyculture with common carp (FAO 2005). It is expected that paddlefish aquaculture may provide additional income for Polish fish farmers, as the fish achieve large size and their eggs may be processed into a valuable caviar (Onders et al. 2005).

At present paddlefish aquaculture in Poland is at experimental stage, and Polish broodstocks are few, small in numbers and

it is possible that they originated from only a few spawners. One of the possible consequences of this situation might be a low level of genetic variation in those stocks, what may result in high probability of inbreeding depression in future generations.

The aim of this study was to optimise protocols of the DNA extraction, PCR amplification of microsatellite loci and genotyping of paddlefish spawners. It is hoped that genetic profiles of mature paddlefish will help their breeders to choose optimal combination of female and male spawners in order to obtain highly heterozygotic progeny.

MATERIALS AND METHODS

Sample collection

Fin-clips were obtained from 100 individuals of the age ranging from 3 to 11 years. Samples were taken from all individuals available in Polish breeding centers (29 from Wąsosze and 24 from Pogórze) and from 47 fish reared in Gorny Tykich (Ukraine). The fin-clipping was practically harmless for fish as the size of each fin-clip was about 10mm². Samples were placed in tubes with 97% ethanol and stored at the temperature of 4°C. Samples from Ukraine were air-dried and mailed to the laboratory in Olsztyn; after their arrival the samples were placed in 97% ethanol and stored at the temperature of 4°C until examination.

DNA extraction

DNA extraction was performed using DNA Genomic Wizard Kit (Promega). However, the Manufacturer's protocol was modified. Tissue samples (1-2mg) were placed in 1.5ml Eppendorf tubes and 200 μ l Nuclei Lysis Solution was added to each tube. Then the samples were incubated for 8-10 hours at the temperature of 65°C. After the incubation, 150 μ l of Protein Precipitation Solution was added to each tube and the samples were centrifuged for 6 minutes (13200 rpm). After centrifugation the supernatant was decanted to new tubes containing 500 μ l of isopropanol; the samples were mixed by hand for 10 minutes and then centrifuged for 4 minutes (13200 rpm). After centrifugation the isopropanol was decanted and 500 μ l of 70% ethanol was added to each tube. Then the samples were again mixed by hand for 10 minutes and centrifuged for 4.5 minutes (13200 rpm). Again after centrifugation the alcohol was decanted and the tubes were placed on thermoblock (65°C) for five minutes in order to evaporate remains of the alcohol. After removing the alcohol the DNA, which precipitated on the bottom of the tubes, was rehydrated in 50 μ l of de-ionized water or Rehydration Solution at 65°C for 2 hours. Samples of the isolated DNA were stored at the temperature of -18°C. Electrophoresis of the extracted DNA (5 μ l DNA solution from each tube) was done in 1.5% agarose gel using TBE buffer (0.120mA and 90V), and the gels were stained with ethidium bromide. Electrophoresis duration varied from 30 to 45 minutes. All pictures of gels were taken using Digital Imaging System and appropriate software. Concentration of double stranded DNA in the samples was checked spectrophotometrically. Samples containing more than 30pg $\cdot\mu$ l⁻¹ of double stranded DNA were chosen for the PCR amplification.

PCR amplification

Amplification of eight microsatellites was accomplished using the PCR technique. Primers were ordered according to Heist et al. (2002) (Table 1). In order to enable genotyping by using Genetic Analysis System CEQ 8000, all primers marked as "F" (forward) were labeled with phosphoramidite dyes (D2-PA, D3-PA and D4-PA).

The fluorescent-labeled primers are light-fragile, therefore all primers and PCR products were protected against light. Fluorescent-labeled primers were stored in a freezer (temperature -20°C) in black boxes. All racks with PCR products were covered with aluminium foil. During the PCR setup the aluminium covers were also used in order to reduce exposure of chemicals for the sunlight. We have used a system of dilutions in order to reduce the number of freezing-thawing cycles. In the first step 10 μ l of stock mixture was diluted with 20 μ l of de-ionized water. Then 10 μ l of this mixture was further diluted with 10 μ l of de-ionized water. This final mixture was used for preparation of the PCR mixture.

Run TAQ polymerase (Aabiot) was used in the amplification of all loci. Properties of this polymerase were similar to GO TAQ DNA polymerase (Promega) and it did not require any addition of MgCl₂. PCR conditions and mixture compositions were optimized for each primer set. PCR reactions were performed in Eppendorf Gradient and Eppendorf Personal thermocyclers.

The PCR stock mixture for all reactions was composed of: 10X polymerase buffer (added to the mixture in concentration supplied with Run TAQ polymerase), nucleotides (concentration 217 μ M $\cdot\mu$ l⁻¹), primers set (concentration 12.5nM $\cdot\mu$ l⁻¹),

Table 1. Microsatellite loci studied in paddlefish (*Polyodon spathula*), their repetitive motifs and primer sequences (according to Heist et al. 2002; May et al. 1997).

Locus name	Repetitive motif	Primer sequences 5'-3'
<i>Psp 12</i>	(GA)	F: ATCTGATACAATCTTCACAGTCC R: GAGTTCAGCTCGCTCTCC
<i>Psp 18</i>	(GA)	F: CTATGTGAGGAATGCCACGAC R: TGACATCACCATGACCTATTGG
<i>Psp 20</i>	(GA)	F: GCATAGTTTTTGGGGATGGC R: ACAACTGCTTCACCGCATTC
<i>Psp 21</i>	(GA)	F: TTCAGCAGGTAGTGAGACAGGCAG R: TCAAGTCCCATCCACTCT
<i>Psp 26</i>	(GT)	F: TCGGTGTTTGTGTGTGTGTATGC R: TGGTTCAGTTTCGCTCATCC
<i>Psp 28</i>	(GA)	F: CAAAGGCATCCCCTACCAC R: GCTGGACAAAAAGTATGGAGTGC
<i>Psp 29</i>	(GCAC)	F: GGGGTCTAATAAAATCCACCGTTC R: TTGCCTTGTGCTCTGTGTTCC
<i>Psp 32</i>	IMP*	F: AATGACTCAGTTGTGTGCTGC R: AAGTGTAGGGAATCTCACCAG

IMP* — imperfect repeat.

polymerase in concentration supplied by the manufacturer ($1\text{u}\cdot\mu\text{l}^{-1}$), and template DNA (concentration $30\text{-}160\text{ng}\cdot\mu\text{l}^{-1}$). Final volume of PCR mixture was $30\mu\text{l}$, and nuclease free water was added to reach this volume. During this step of the study

the PCR enhancers and mineral oil were not used. Before splitting stock mixtures to sample tubes, the samples were vortexed for 30 seconds. Optimal compositions of the PCR chemicals are given in Table 2.

Table 2. Components of PCR mixture and annealing temperatures used in studying microsatellites of padlefish (*Polyodon spathula*). In each case the amount of polymerase buffer was $3\mu\text{l}$ and the total volume of reaction mixture was $30\mu\text{l}$.

Components and temperature	Locus: <i>Psp</i>							
	12	18	20	21	26	28	29	32
Nucleotide mix (μl)	2.0	2.0	1.6	2.0	1.6	2.0	2.0	2.0
Upstream (F) primer (μl)	0.6	0.4	0.6	0.4	0.6	0.6	0.6	0.4
Downstream (R) primer (μl)	0.6	0.4	0.6	0.4	0.6	0.6	0.6	0.4
Nuclease free water (μl)	22.2	22.5	22.2	22.8	22.6	22.5	22.4	22.8
Polymerase (μl)	0.6	0.4	0.8	0.4	0.6	0.4	0.4	0.4
Template DNA (μl)	1.0	1.3	0.6	1.0	1.0	1.2	1.0	1.0
Annealing temperature ($^{\circ}\text{C}$)	59.0	59.0	61.0	62.0	63.0	59.0	60.0	62.0

A typical PCR program of amplification consisted of 37 cycles. In case of easily amplifiable loci, such as: *Psp 12*, *Psp 20*, *Psp 29* and *Psp 32*, the number of cycles was reduced to 35. The temperature and the time of an initial denaturation, and those of final extension, followed guidelines of Promega GO TAQ DNA polymerase. The extension duration was 35 seconds at the temperature of 75°C for all

investigated microsatellites. Annealing temperature was specific for each microsatellite (Table 2), and duration of this step was 35 seconds for all loci. Quality of PCR product ($5\mu\text{l}$ PCR product from each sample) was examined by electrophoresis (1.5% agarose gel, 0.120mA and 90V , 35 minutes) and the gels were stained with ethidium bromide (Figure 1 and 2).

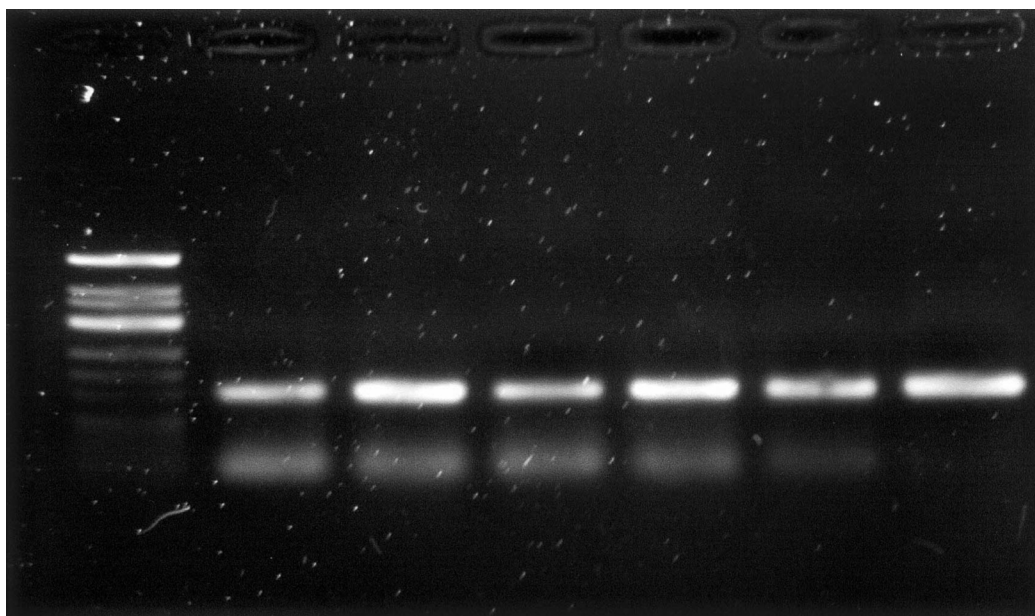


Figure 1. Agarose gel with PCR products of studied micro-satellite loci of *Polyodon spathula*. Lanes: 1 – molecular weight marker 50bp DNA Step Ladder, 2 – locus *Psp 28*, 3 – locus *Psp 26*, 4 – locus *Psp 21*, 5 – locus *Psp 20*, 6 – locus *Psp 18*, 7 – locus *Psp 12*.

Genotyping

Genotyping was performed using Genetic Analysis System CEQ 8000. Eight investigated loci have been arranged in three sets according to the kinds of fluorescent-labeled

primers and to the expected length of their PCR products. Set No. 1 consisted of loci *Psp 18*, *Psp 12* and *Psp 28*, set No. 2 consisted of loci *Psp 26*, *Psp 21* and *Psp 20*, and set No. 3 consisted of loci *Psp 32* and *Psp 29*.

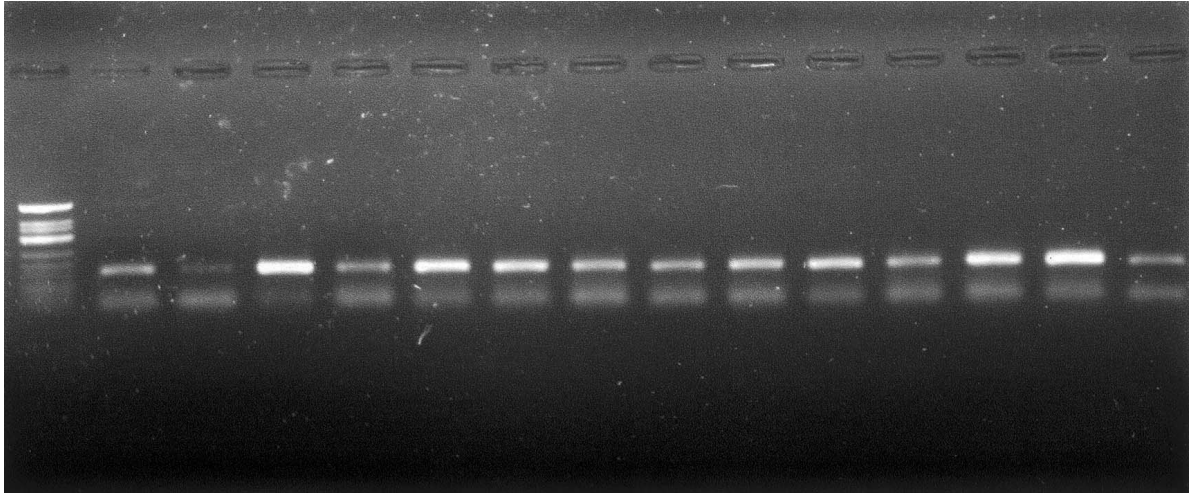


Figure 2. Agarose gel with PCR products of locus *Psp 20* of *Polyodon spathula*. Lanes: 1 – molecular weight marker, 2-15 – locus *Psp 20*.

RESULTS

DNA was successfully extracted from all tissue samples. Long (8-10 hours) incubation time of the tissues in Nuclei Lysis solution resulted in a better lysis of cell membranes and significantly improved DNA extraction procedure. Modified DNA extraction method applied to the tissue material taken from fish of the age 7 years or more, usually secured better results than that obtained by use of manufacturer's protocol applied to tissues taken from younger fishes. DNA from the tissues that were air-dried and mailed from Ukraine to Olsztyn was also successfully extracted, and this method of shipping fin samples did not affect results of the PCR amplification.

An amount of 30-160ng template DNA was sufficient for successful amplification of studied microsatellites. The amplification of locus *Ls 57* from paddlefish genome was possible by using primers designed for lake sturgeon. However, reaction conditions and composition of mixtures used in the PCR still require optimization before genotyping of this locus is possible. Amplification of other loci was successful while using primer sequences and the thermal profiles described above.

Protection of primers and PCR products against sunlight proved effective, and only a few samples were not scorable because of the burned out phosphoramidate labels of primers. All studied microsatellite loci were polymorphic and the alleles specific for each population were observed. Most of the loci were disomic, and only the locus *Psp 29* was tetrasomic. Because of the occurrence of many additional unspecific products, genotyping of some samples containing locus *Psp 12* was unsure.

DISCUSSION

Estimation of genetic variation based on polymorphism of microsatellite loci was useful in studying genetic variation in sturgeon populations. In this paper we report the results of the optimisation of the extraction and amplification procedures, which enabled genotyping of microsatellite loci by using Genetic Analysis System CEQ 8000 (Figure 3).

The transport of the air-dried fin tissues was optimal in a situation when sending fresh samples preserved in alcohol was not possible. This approach did not affect neither the DNA extraction nor the microsatellites amplification. We also observed that in most cases the tissues taken from older individuals secured better extraction results than those coming from the younger ones.

Annealing temperatures applied in this study (Table 2) were different from those described by Heist et al. (2002); we suppose that this was because in our laboratory we used sets of chemicals that were slightly different to those used by Heist et al. (2002). Also, the amounts of primers were very important for successful microsatellite amplification. Too much or too little amounts of primers usually arrested the PCR. Conditions of PCR and composition of reaction mixtures might still require some optimization, as during the genotyping of the locus *Psp 29*, in several samples single-nucleotide splitting of PCR products occurred. Those splits were easy to identify and did not affect the results, and one

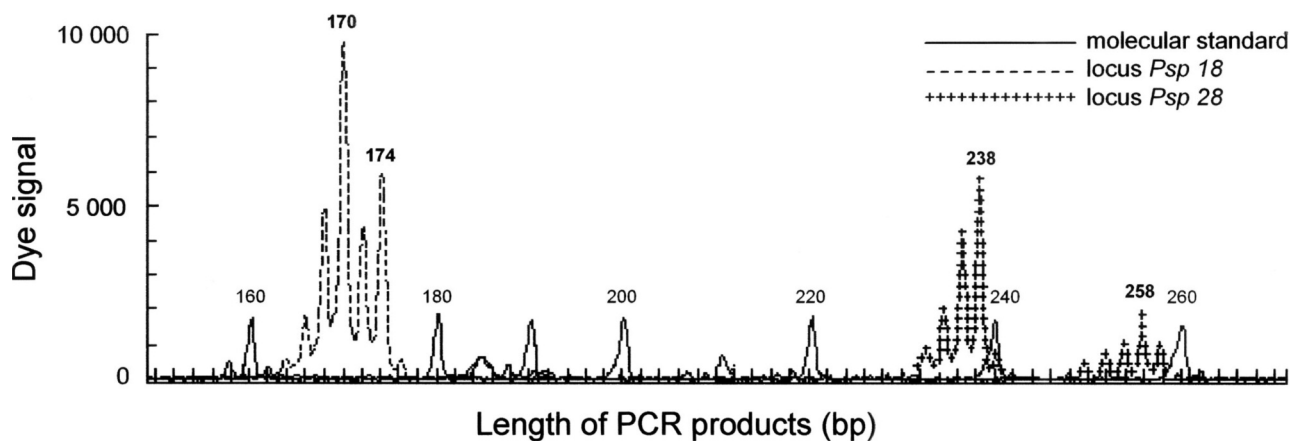


Figure 3. Example of genotyping results of *Polyodon spathula* microsatellite DNA obtained by using Genetic Analysis System CEQ 8000. Amplified DNA fragments contained loci *Psp 18* (allele size 170 and 174 bp) and *Psp 28* (238 and 258 bp). Molecular standard: SS 400.

may expect that they could be eliminated by further optimization of the PCR conditions. Optimisation of PCR conditions might also eliminate unspecific products occurring in case of the locus *Psp 12*.

This work constitutes a technical report, describing the procedures rather than the results obtained by applying those methods (the results of our studies, however, are described elsewhere (Kaczmarczyk et al. 2008)). We tested our methods for samples taken from paddlefish individuals of different age (from 1 to 13 years). We conclude that the optimized protocols could provide guidelines to those who start their studies on the paddlefish microsatellite DNA. The chemicals used in our studies were standard ones and available on the market. As the development of an effective method of the DNA extraction and the amplification of the microsatellites using the PCR technique for a “new” species requires a lot of time and effort, we hope that those who will follow our observations will find them helpful in optimizing their own procedures.

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