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VERIFICATION OF MEIOTIC GYNOGENESIS IN BROOK TROUT, *SALVELINUS FONTINALIS* (MITCHILL) USING MICROSATELLITE DNA MARKERS

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ABSTRACT. The microsatellite DNA technique was applied to assess rapidly the overall success of treatments designed to induce gynogenesis. The results of gynogenesis were verified based on the analysis of two microsatellite loci (*Sfo-296* and *Sfo-262*). Three brook trout, *Salvelinus fontinalis* (Mitchill), females, one brown trout, *Salmo trutta morpha fario* L., male, and their offspring (30 individuals from each female) were studied. Locus *Sfo-292* was monomorphic in the studied brook trout offspring while *Sfo-262* was polymorphic. The paternal genotype was absent in the studied gynogenotes. This simple microsatellite-based technique could be generally applicable for the verification of gynogenesis in brook trout when the UV-irradiated milt of brown trout is used.

Key words: BROOK TROUT, GENETIC MARKERS, GYNOGENESIS, MICROSATELLITE DNA, *SALVELINUS FONTINALIS*

INTRODUCTION

The brook trout, *Salvelinus fontinalis* (Mitchill), is a northeastern American species that was introduced to Poland at the end of the nineteenth century. Brook trout had been reared since 1965 in the hatchery of Department of River Fisheries (Inland Fisheries Institute, Olsztyn) and then it was transferred to Rutki (near Gdansk). Brook trout is a farmed salmonid species that is far less popular than the rainbow trout. This species has been the subject of genome manipulation including triploidization (Woznicki and Kuzminski 2002, Własow et al. 2004), intergeneric hybridization with other salmonids (Dorson et al. 1991, Goryczko et al. 1991, Goryczko et al. 1992), and also gynogenesis, but only isozyme loci analysis was used for the genetic identification of

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manipulated individuals (Fujino et al. 1989, Arai et al. 1991, Galbreath and Stocks 1999, Galbreath et al. 2003).

As one of the most powerful genetic markers, microsatellites have been widely used in genetics and related studies in recent years. Microsatellite DNA analysis is a valuable tool for the identification of gynogenetic offspring. This method would greatly facilitate molecular identifications in genome manipulations, e.g. induction of polyploids, gynogenesis, androgenesis, and the performance of controlled mating or hybridization. Microsatellite DNA, or short sequence repeat (SSR), is a PCR-based and co-dominant DNA marker (Tautz 1989). Highly polymorphic SSRs have great potential as genetic markers in aquaculture. They have proven to be particularly valuable for parentage, stock discrimination, population genetics, and genome mapping due to high levels of polymorphism (O'Connell and Wright 1997). However, the isolation of microsatellites remains a somewhat tedious task. The conservation of flanking regions in microsatellites has been reported in many animals (Schlotterer et al. 1991, Angers and Bernatchez 1996, O'Connell and Wright 1997), and this facilitates cross-species amplification in a new species. Although a number of microsatellites have been developed for brook trout (Angers et al. 1995, Angers and Bernatchez 1996, Perry et al. 2005), there is no information about diagnosing their gynogenetic offspring using microsatellite loci. In the present work the diagnostic analysis of brook trout following gynogenesis was demonstrated using two microsatellite loci (*Sfo-262* and *Sfo-292*) with practical applications in the research of genetics and breeding. The objective of the present study was to confirm the success of brook trout meiotic gynogenesis using noninvasive microsatellite DNA analysis.

MATERIALS AND METHODS

Artificial gynogenesis was induced in brook trout using UV-irradiated sperm from brown trout, *Salmo trutta* m. *fario* L. The milt was diluted 1: 40 in the seminal plasma of the brown trout. Samples were irradiated in Petri dishes under a UV lamp (Phillips TUV 30W) for 10 minutes. The distance between the lamp and the sperm surface was 20 cm. Samples of about 1000 eggs from three brook trout females were inseminated with the irradiated brown trout sperm and pressure shocked (62.05 MPa, for 5 minutes) 40 minutes after fertilization at 10°C. The treated eggs were incubated in a

California incubator at 10°C. After hatching, the larvae were counted and the percentage of viable gynogenotes was calculated based on control hatching.

A sample of 90 gynogenetic offspring from three females, three maternal individuals (Gyno1, Gyno2, Gyno3), a control group of 30 individuals of brook trout from the Rutki stock, and a brown trout male (no. 94), which was the milt donor, were analyzed using the two microsatellite markers.

Fins from these fish were clipped at the farm and stored in 96% ethanol. Genomic DNA was extracted using the Chelex 100 method (Walsh et al. 1991) from 124 individuals of the following brook trout groups from the Rutki (Poland) broodstock: control (n = 30), gyno1 (n = 30), gyno2 (n = 30), and gyno3 (n = 30) and of four parental individuals: Gyno1, Gyno2, Gyno3 (maternal) and brown trout (paternal). Two microsatellite loci were analyzed: *Sfo-262* and *Sfo-292* (Perry et al. 2005). Reaction mixes were prepared in a total volume of 15 µl with a 0.5 µl DNA template, 1.5 µl PCR reaction buffer (50 mM KCl, pH 8.5; Triton X-100), 0.5 µl of each primer, 1 µl (500 µM) each of deoxynucleotide triphosphate (dNTP), 0.8 µl $MgCl_2$, and 0.25 µl Run DNA polymerase (A&A BIOTECHNOLOGY, Poland). Re-distilled water was used to bring the reaction mixture to the desired final volume. Amplification was conducted with a Perkin Elmer thermocycler Gene Amp-System 9600 (PE-Applied Biosystem, California, USA), with initial denaturation at 94°C for 5 min, followed by 30 amplification cycles (94°C, 1 min; 60°C, 30 s; 72°C, 30 s), and final elongation at 72°C for 5 min. Aliquots containing PCR products and reaction buffer were electrophoresed using 6% polyacrylamide gel, and DNA bands were then visualized with the silver staining method (Tegelström 1986). Electrophoresis was conducted on a Bio-Rad SequiGen Sequencing Cell-system, and the gel size was 38 × 30 cm. The amplified fragments were sized by comparing them to molecular standards ϕX 174 DNA/Hinf I DNA Step Ladder (Promega) and 25 bp DNA Step Ladder (Promega).

RESULTS

The application of UV-irradiated brown trout spermatozoa and pressure shock was effective in producing viable brook trout gynogenotes. The survival rate of gynogenotes was 9% for the Gyno3 female, 7% for the Gyno2 female, and 5% for the Gyno1 female, while 100% mortality was observed in the haploid control.

The total number of alleles observed at microsatellite locus *Sfo-262* in the studied fish groups was three: 274, 289, and 297 base pairs (bp). The frequencies of particular alleles, 0.02, 0.53 and 0.45, respectively, were observed in the control group. One of the maternal individuals was homozygous with the presence of 297 bp allele (Gyno1), while two other females were heterozygous (Gyno2 with 289 bp and 297 bp alleles and Gyno3 with 274 bp and 289 bp alleles).

In the paternal individual of brown trout the allele 254 bp was observed at locus *Sfo-262* (Fig. 1). Locus *Sfo-292* was monomorphic for all the studied maternal individuals and gynogenetic of the offspring, and allele 187 bp was observed. In the paternal individual, two alleles (191 and 215) were observed at locus *Sfo-292*. The results of molecular identification for gynogenetic offspring in brook trout are presented in Figs. 1 and 2.

A single PCR amplification indicated unambiguously that offspring from artificial gynogenesis originated from the corresponding maternal individual. Two tested microsatellite primer pairs were positively amplified in four groups and four parental specimens. The results show maternal-specific patterns, with the number of scorable bands ranging from 1 to 2 on polyacrylamide gel. No paternal genotype was observed in the three tested gynogenetic groups. There were also no specimens with different phenotype coloration specific to brook trout x brown trout (tiger trout) in any of the three gynogenetic groups.

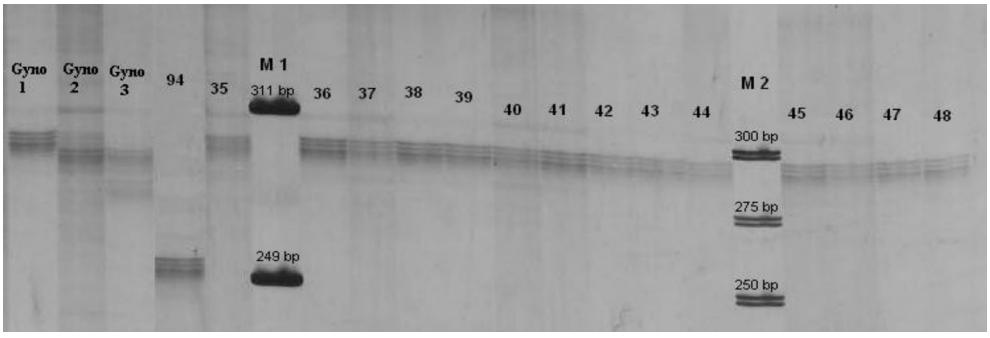

Fig. 1. Demonstration of the exclusive maternal contribution to gynogenetic offspring of brook trout using diagnostic microsatellite locus *Sfo-262*. The electrophoretic patterns show those of three maternal individuals in lanes: 1, 2 and 3; the paternal individual in lane 4; a sample of 14 gynogenetic offspring of the Gyno1 female in lanes 5, 7-15 and 17-20. Additionally, lanes 6 and 16 contain two standard size markers (M 1 – ϕX174, Promega and M 2-25 bp, Promega).

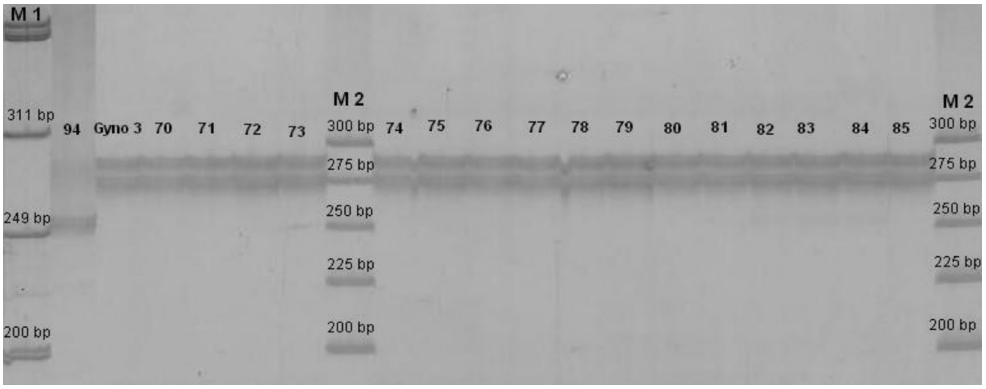

Fig. 2. Demonstration of exclusive maternal contribution to gynogenetic offspring of brook trout using the diagnostic microsatellite locus *Sfo-262*. The electrophoretic patterns show the standard size marker (M 1 – ϕX174, Promega and M 2-25 bp, Promega) in lanes: 1, 8, and 21; the paternal individual in lane 2; the Gyno3 maternal individual in lane 3; and 16 gynogenetic offspring of the Gyno3 female in lanes 4-7 and 9-20-.

DISCUSSION

The traditional method of producing pure lines by means of selection and inbreeding is an extremely slow process. Gynogenesis allows for the production of inbred lines or clones in the second generation (Purdom et al. 1985, Kobayashi et al. 1994, Sarder et al. 1999). Ploidy manipulation reported for many species of fish and diploid gynogenesis has been successfully induced and used as a powerful tool for examining sex determination (Avtalion and Don 1990, Devlin and Nagahama 2002) and the production of inbred lines, mono-sexual broods, or clones (Pandian and Koteeswaran 1998, Arai 2001). The artificial induction of gynogenesis in salmonids is of interest to both commercial aquaculturists and researchers investigating the developmental biology and genetics of fish.

The development of highly variable genetic markers in recent years has provided a source of polymorphism necessary to achieve genetic identification in fish. Parentage and pedigree analysis is currently possible in many fish species by using DNA markers like minisatellites and microsatellites (O'Reilly and Wright 1995, Carvalho and Hauser 1998, Ferguson and Danzmann 1998). The main objective of the current study was to develop a method that would contribute to the genetic verification of gynogenesis in brook trout. In this regard, developing a reliable method for the verification of gynogenesis in brook trout will apparently be useful in ensuing genome manipulations.

The present paper describes a technique based on microsatellites *Sfo-262* and *Sfo-292* to rapidly screen putative gynogenetic offspring.

One of the important problems of microsatellite DNA analyses is the slippage of Taq polymerase during PCR, which results in the addition of an extra base at the end of the amplified fragments (Hu 1993). These additional bands are referred to as stutter or shadow bands (such bands are shown in Fig. 1). Scoring the gel can still be carried out unambiguously under the assumption that the true band that reflects the actual allele is the most intense one that is located close to the neighboring group of its stutter bands.

Considering the results of the present study, microsatellite loci *Sfo-262* and *Sfo-292* should have be the reliable loci for diagnosis after gynogenesis. In addition, locus *Sfo-262* can also potentially be the dominant marker for studies on the genetic diversity of different brook trout stocks.

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STRESZCZENIE

WERYFIKACJA EFEKTÓW GYNOGENEZY MEJOTYCZNEJ PSTRĄGA ŹRÓDLANEGO *SALVELINUS FONTINALIS* (MITCHILL) PRZY ZASTOSOWANIU MARKERÓW MIKROSATELITARNEGO DNA

Opracowano technikę umożliwiającą szybką weryfikację efektów zabiegu indukowanej gynogenezy u pstrąga źródlanego, *Salvelinus fontinalis* (Mitchill). Wykorzystano dwie pary starterów mikrosatelitarnego DNA: lokus *Sfo-292* oraz lokus *Sfo-262*. Badania przeprowadzono na trzech samicach pstrąga źródlanego, jednym samcu pstrąga potokowego, *Salmo trutta* m. *fario* L. oraz gynogenetycznym potomstwie tych ryb (30 osobników potomnych od każdej z samic). Lokus *Sfo-292* był monomorficzny, podczas gdy lokus *Sfo-262* był polimorficzny. Nie stwierdzono obecności ojcowskiego genotypu w żadnej z trzech badanych grup potomstwa gynogenetycznego pstrąga źródlanego. Ta prosta metoda, oparta na analizie mikrosatelitarnego DNA, może mieć zastosowanie w diagnozowaniu efektów gynogenezy pstrąga źródlanego wykonanej przy zastosowaniu inaktywowanego mlecza pstrąga potokowego lub troci *Salmo trutta* m. *trutta* L.