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THE RAPID DIAGNOSTICS OF SEX OF SALMONIDS USING DNA-MARKERS

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Based on nucleotide sequences of sex-specific DNA-markers of salmonid fishes the oligonucleotide primers for polymerase chain reaction were selected with purpose on rapid diagnostic of sex in rainbow trout *Onchorhynchus mykiss*, brown trout *Salmo trutta*, huchen *Hucho hucho* and grayling *Thymallus thymallus*. The specify of amplification was determined by nucleotide sequence analysis of PCR-products. All amplified fragments were referred to sex-specific locuses of Y chromosomes in males of investigated fish species. The PCR-products were in size of 880, 607, 521 and 558 for rainbow trout, brown trout, grayling and huchen respectively. Thus the sex determination in above mentioned fish species and identification of genotypic males under process of hormonal sex reversion can be provided using conventional PCR. Present method relates to rapid diagnostics because the data analysis and return of results back to fish farm take one single day.

Key words: DNA-markers, PCR diagnostics, sex reversion, salmon raising.

The burning development of molecular biology in the end of 20th century, especially genomics and proteomics, promoted a creation of the new research methods in aquaculture that are directed to accelerate acquiring of fishery products and improving facilities of fish farming cultivation in the world generally. The newest technologies are applied successfully in investigations of fish embryonic development, sexual differentiation, growth hormones impact and resistance to infectious diseases [1].

Monosex or sterile populations of fish at the time of modern aquaculture are economically desirable for a variety of reasons. Firstly, the male grows faster in some species and the female faster in other species. In this case, monosex culture of the faster-growing sex can increase production. Secondly, some species of fish mature at small sizes and young ages prior to the desired time of harvest. Thirdly, the sex differences may also exist for flesh quality and carcass yield, because as the fish become sexually mature, growth rate slows and carcass yield decreases. Also, some studies showed that sexual dimorphism could also exist for other economic traits, such as disease resistance or tolerance of poor water quality [2, 3]. Therefore fish producers have a great interest in the possibility to raise the generation only females or only males.

Several techniques are available for producing monosex or sterile populations of fish for aquaculture. Manual sexing, sterilization, hybridization, gynogenesis, androgenesis and polyploidy. Monosex populations can be produced by direct hormonal sex reversal. Phenotypic sex can be altered by administration of sex hormones, oestrogens or androgens, at the critical period of sex determination. The development of fish makes them conducive to the manipulation of their sex. Although the male or female genotype is established at fertilization, phenotypic sex determination occurs later in development. Sex reversal is a manipulation for controlling reproduction in fish and ultimately improving the rate of growth [4].

Accredited in EU method of indirect feminization provides a step of “neomales” selection before obtaining 100% female progeny. The neomales are males that have females (XX) genotype in which under influence of androgens (17-α methyltestosterone) the testes have been developed. In these phenotypic males mature sperm are formed and able to fertilize eggs. However, the genotype of individuals are stored, and therefore all of inverted males contain in germ cells only the X sex chromosomes. In case of intercross the native, not hormone-treated, females with neomales, the monosex populations of females are got in the first generation (F1). A key step

for the selection of neomales in F0 generation is to identify genotypic males (XY), which certainly should not take part in the next crossing with a purpose to providing 100% females progeny.

The rainbow trout *O. mykiss*, brown trout *S. trutta*, huchen *H. hucho* and grayling *T. thymallus* are the most valuable salmonids of rivers by Danube basin in Carpathians. These fish species are traditional of salmon breeding in Ukraine for the purposes of caviar — commodity production. Artificial breeding of these fish species using modern biotechnology will increase their populations. The hormonal sex reversal is a one of the research areas of biotechnology in aquaculture. This method includes an essential step of searching the sex-specific DNA-markers that are required for rapid diagnostic of fish sex. Therefore the goals of our research were to analyze the nucleotide sequences of Y chromosome in salmonids, to chose the fragments for specific oligonucleotide primers selection and to develop the method based on the conventional PCR for rapid diagnostic of sex in rainbow trout, brown trout, huchen and grayling reared in Ukraine.

Material and Methods

DNA extraction. The DNA was extracted from fins of sex-maturated rainbow trout (♂ $n = 4$, ♀ $n = 4$), brown trout (♂ $n = 3$, ♀ $n = 3$), huchen (♂ $n = 4$, ♀ $n = 4$) and grayling (♂ $n = 4$, ♀ $n = 4$) using phenol-chloroform method. The fins were sampled *in vivo*. All fins were independently homogenized in PBS (137 mM NaCl, 2,7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7,4) using IKA T18 Ultra Turrex (Germany). Then the lysis buffer (10 mM Tris-HCl, pH=8,0, 0,1 M NaCl, 25 mM ЕДТА, 0,5% SDS) and Proteinase K were added. Incubation lasted 2 hours at 37 °C. Genomic DNA was extracted by the 25:24:1 phenol:chloroform:isoamyl alcohol (IAA) mixture using a microcentrifuge Ependorf (Germany) at 13 000 rpm for 5 min. After this a 0,1 volume of 3 M sodium acetate and 2,5 volumes of absolute ethanol were added to supernatant. The alcohol precipitation was carried out at room temperature for 1 h. The DNA was pelleted by centrifugation at 13000 rpm for 10 min. The DNA pellets were rinsed in 70% ethanol mixture and dissolved in nuclease free water [5]. The DNA concentration and its quality were measured by spectroscopy in APEL PD-303 UV (Japan).

Oligonucleotide primers. The analysis of nucleotide sequences of sex-specific DNA fragments in Y-chromosomes of the salmonids, available in the National Centre of Biotechnological Information (NCBI) was conducted in the on-line service BLAST (www.ncbi.nim.nih.gov) and software of MEGA 6.0. For the primers design and evaluation of oligonucleotides possible secondary structure or self-complementarity the software of Vector NTI 11 was used.

PCR assay. The 96 Universal Gradient PeqStar thermocycler (PEQLAB, Germany) was used for DNA amplification. The reagents used for PCR were from ThermoScientific (USA). The PCR mixture (prepared on ice) contained 1 µl of DNA sample, 12,5 µl of DreamTaq™ Green PCR Master Mix (2X) (ThermoScientific), 1 µl of each oligonucleotide primer (20 pmol/µl) from Metabion (Germany) and nuclease free water up to total volume of 25 µl. The DNA amplification included a cycle of initiative denaturation at 94 °C for 3 min and followed 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 1 min. A final cycle of extension was at 72 °C for 7 min. After the PCR, all products were analyzed in electrophoresis using 2.0% agarose gel and TAE buffer (40 mM Tris-HCl, 20 mM acetic acid, 1 mM EDTA). The results of electrophoresis was visualized in UV transilluminator.

Sequencing. For extraction of DNA from agarose gel the Silica Bead DNA Extraction Kit (ThermoScientific, USA) was used. Sequencing was performed on a 3130 Genetic Analyzer (Applied Biosystems, USA) using the BigDye® Terminator v3.1 Cycle Sequencing Kit. The sequences were analyzed using software of Sequencing Analysis (Applied Biosystems).

The DNA alignments of rainbow trout, brown trout, huchen and grayling were performed in MEGA 6.0 software and BLASTN using the ClustalW algorithm. All available DNA sequences of other salmonids were obtained from NCBI database.

Results and Discussion

The results of our analysis comprised multi alignment of DNA fragments of sex-specific sequences in fish species from *Salmonidae* family showed that high homology of Y-chromosome is characteristic only for fish belonging to the same genus. For example, fish species of *Salmo*, *Oncorhynchus* and *Salvelinus* genera do not share complete homology in Y chromosome sequences, but have short highly conserved DNA fragments which are common for all salmonids [6]. Similar sequences with

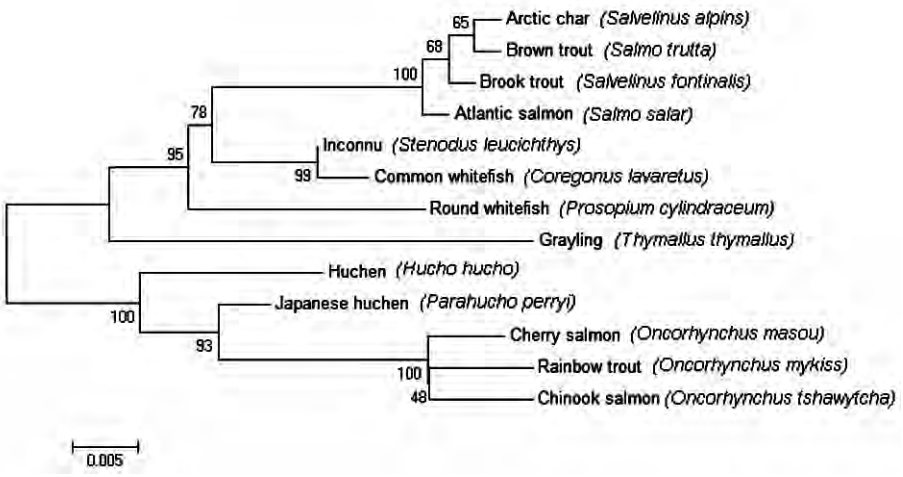

Fig. 1. **The phylogenetic analysis of sdY loci in salmonids**
The tree was generated by means of Neighbor-joining algorithm in MEGA 6.0

identity of 90% were identified in fish from *Oncorhynchus* genus, such as chinook salmon *O. tshawytcha,* coho salmon *O. kisutch* and rainbow trout *O. mykiss*. The traced DNA fragments were in size from 2000 to 4000 base pairs (bp) [7, 8]. These specific sequences are characteristic only for males and lacks in DNA of females. In addition to the genus *Oncorhynchus*, similar sequences were found in Atlantic salmon *Salmo salar*, brown trout *S. trutta*, brook trout *Salvelinus fontinalis* and other salmonids [9]. These loci were named as sexually dimorphic on Y chromosome (sdY). The computation of all available in the NCBI database DNA sequences of sdY loci by means of Neighbor-joining algorithm in MEGA 6.0 revealed that presence of these highly conserved sequences in almost all salmon species except genus *Coregonus* (Fig. 1).

Our results showed that specific oligonucleotide primers for sdY loci successfully amplified single products in expected size for males of rainbow trout, brown trout, huchen and grayling. The PCR products ranged in length from 520 to 880 bp and depended on oligonucleotide primers combination (Table).

The selected oligonucleotide primers were tested in the reaction with DNAs of rainbow trout, brown trout, huchen and grayling males and females. The results of experiment confirmed that PCR products for rainbow trout were present only in DNA samples of males with size of 880 bp. (Fig. 2). The specific amplicons for males of brown trout, huchen and grayling were in size of 607, 558 and 521 bp respectively (Fig. 3–5).

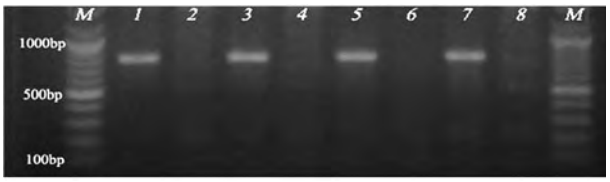

Fig. 2. **The amplification of sdY loci fragment in rainbow trout *O. mykiss*:**
odd numbers — males; even — females;
M — Quick-Load DNA Ladder 100 bp (BioLabs)

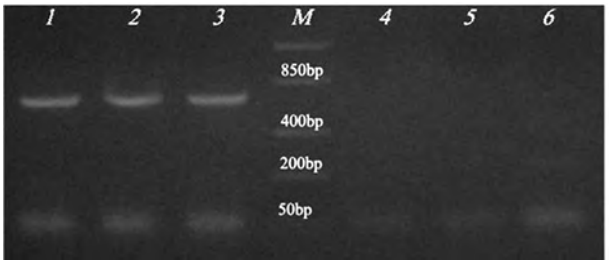

Fig. 3. **The amplification of sdY loci fragment in brown trout *S. trutta*:**
1–3 — males; *4–6* — females; *M* — FastRuler Low Range DNA Ladder (ThermoScientific)

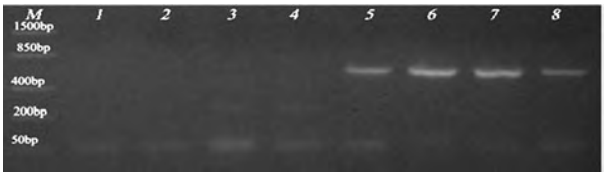

Fig. 4. **The amplification of sdY loci fragment in huchen *H. hucho*:**
1–4 — males; *5–8* — females; *M* — FastRuler Low Range DNA Ladder (ThermoScientific)

The oligonucleotide primers for sex-specific loci in Y chromosome of rainbow trout, brook trout, huchen and grayling

Species	Oligonucleotide primers	T_a, °C	Product, bp
Rainbow trout *Oncorhynchus mykiss*	5′-GTTCATATGCCAGGCTCAAC-3′ 5′-CGATTAGAAAGGCCTGCTTG-3′	58	880
Brown trout *Salmo trutta*	5′-GTGGAGTACTGCGAAGATGAG-3′ 5′-CTTAAAACCACTCCACCCTCC-3′	63	607
Grayling *Thymallus thymallus*	5′-ATGGCTGACAGAGAGGCCAGA-3′ 5′-CTTAAAACCACTCCACCCTCC-3′	65	521
Huchen *Hucho hucho*	5′-ATGGCTGACAGAGAGGCCAGA-3′ 5′-GCATAGATGCCTTCCCTAGA-3′	67	558

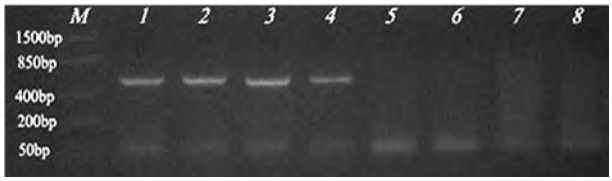

Fig. 5. **The amplification of sdY loci fragment in grayling *T. thymallus*:**
1–4 — males; *5–8* — females; *M* — FastRuler Low Range DNA Ladder (ThermoScientific)

The specificity of amplification was verified by sequencing of PCR-products. Our results of sequencing confirmed that amplified fragments referred to sex-specific Y-chromosome loci of salmon species. It should be noted that oligonucleotide primers used to amplify sex-specific loci of sdY in brown trout, huchen and grayling are interchangeable. The combination of primers resulted in the products formation of different lengths ranging from 200 to 900 bp. However, in some variants PCR products appeared in females. Therefore, the table shows the pairs of oligonucleotide primers for males identification only. Such structure of the Y-chromosome of these fish species indicates about highly conserved sex-specific sdY locus and its presence in various members of *Salmonidae* family.

Thus, as a result of precise oligonucleotide primers selection the PCR based method for rapid identification of males in rainbow trout, brown trout, huchen and grayling was developed. At the step of neomales detection during hormonal sex reversal this method will allow to identify the genotypic males (XY) in the experimental group, which should not be used in the next mating with purpose of producing 100% females progeny. Given the fact that for production of red caviar the males are not necessary, and the identification of sex in salmonids is possible only after a few years, consequently the issue of gender diagnosis is extremely important. For example, the possibility of rapid identification of huchen males and their rejection reduces the cost for females cultivation up to 30%.

Often X and Y chromosomes in some species such as Japanese rice fish *Oryzias latipes*, have similar morphology and carry the same genetic information [10]. Ittura et al. (2001) using fluorescence *in situ* hybridization showed that DNA-marker 5S rDNA in rainbow trout reacted with sex chromosomes on two loci in both males and females [11]. This indicates the similarity of X and Y chromosomes, and unlike birds and mammals, early stage of differentiation of Y chromosome in fish makes them important in study of the sex chromosomes evolution.

In 10% of fish species, including salmonids, the sex-specific markers were identified [12]. The sex in these fish species can be quickly determined using conventional PCR assay. However, in some fish species the sex-specific DNA markers were not found. In particular, the results of experiments which were obtained in laboratories from Germany, France and Italy, indicated a lack of sex-specific DNA-markers in four sturgeon species (*Acipenser baerii, A. naccarii, A. gueldenstaedtii, A. ruthenus*). Therefore the rapid diagnosis of sex by PCR technique in sturgeons is impossible [13].

One sex grows faster than the other in most fish species. Channel catfish *Ictalurus punctatus* and tilapia *Oreochromis niloticus* males grow faster than females whereas the opposite relationship exists for grass carp, *Ctenopharyngodon idella*, rainbow trout

and other salmonids and cyprinids, where females grow faster than males [14]. Females of salmonids have the advantages over males in breeding, as characterized by late maturation, rapid growth rates and improved taste of meat. Manual separation of the sexes requires the least amount of technology for monosex culture, but it is extremely wasteful, tedious, inefficient and has the potential for mistakes [15]. Before manual separation the fingerlings must be grown to a size large enough to visually determine sex, but in this way half of the fingerling production being wasted. Unnecessary sex should be separated as soon as possible to prevent uncontrolled reproduction. Therefore, methods based on DNA technologies are able to identify the sex at the early stages of ontogenesis and have significant advantages over manual separation of fish.

Thus, using conventional PCR we have developed the method of sex identification in rainbow trout, brown trout, huchen and grayling. It allows to diagnose the sex of mentioned above fish species at early stage of the sex reversion process and cull genotypic males with purpose to reduce the cost of their maintenance.

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ЕКСПРЕС-ДІАГНОСТИКА СТАТІ ЛОСОСЕВИХ РИБ З ВИКОРИСТАННЯМ ДНК-МАРКЕРІВ

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На основі нуклеотидних послідовностей статевоспецифічних ДНК-маркерів лососевих риб було підібрано олігонуклеотидні праймери для полімеразної ланцюгової реакції з метою експрес-діагностики статі у райдужної форелі *(Onchorhynchus mykiss)*, струмкової форелі *(Salmo trutta)*, дунайського лосося (*Hucho hucho)* та харіуса європейського *(Thymallus thymallus)*. Специфічність ампліфікації перевірено нуклеотидним аналізом послідовності продуктів ПЛР. Усі ампліфіковані фрагменти відповідали статевоспецифічним локусам Y-хромосоми самців досліджуваних видів риб. Розміри ампліконів для райдужної форелі, струмкової форелі, харіуса та дунайського лосося становили 880, 607, 521 та 558 пар нуклеотидів відповідно. Таким чином, стать зазначених видів риб може бути визначена за допомогою звичайної ПЛР, що уможливить ідентифікацію генотипових самців під час процесу гормональної реверсії статі у риб. Цей метод належить до експрес-діагностики, оскільки аналіз даних та повернення результатів до рибного господарства здійснюються упродовж однієї доби.

Ключові слова: ДНК-маркери, ПЛР-діагностика, реверсія статі, лососівництво.

ЭКСПРЕСС-ДИАГНОСТИКА ПОЛА ЛОСОСЕВЫХ РЫБ С ИСПОЛЬЗОВАНИЕМ ДНК-МАРКЕРОВ

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На основе нуклеотидных последовательностей полспецифических ДНК-маркеров лососевых рыб были подобраны олигонуклеотидные праймеры для полимеразной цепной реакции с целью экспресс-диагностики пола у радужной форели *Onchorhynchus mykiss*, ручьевой форели *Salmo trutta*, дунайского лосося *Hucho hucho* и хариуса европейского *Thymallus thymallus*. Специфичность амплификации проверена нуклеотидным анализом последовательности продуктов ПЦР. Все амплифицированные фрагменты отвечали полспецифическим локусам Y-хромосомы самцов исследуемых видов рыб. Размеры амликонов для радужной форели, ручьевой форели, хариуса и дунайского лосося составляли 880, 607, 521 и 558 пар нуклеотидов соответственно. Таким образом, пол вышеуказанных видов рыб может быть определен с помощью обычной ПЦР, что позволит идентифицировать генотипических самцов во время процесса гормональной реверсии пола у рыб. Этот метод относится к экспресс-диагностике, поскольку анализ данных и возвращение результатов в рыбное хозяйство осуществляются в течение одних суток.

Ключевые слова: маркеры ДНК, ПЦР-диагностика, реверсия пола, лососеводство.