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Sex Determination Systems Based on PCR for Salmonids Reared in Ukraine

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Aims. To analyse highly conserved male-specific sequences in Y chromosome of salmonid species and develop the conventional polymerase chain reaction (PCR) for rapid identification of fish sex. **Methods**. DNA sequence analysis, phylogenetic analysis, DNA extraction, primers design, PCR and sequencing were used. **Results.** Using the data from NCBI GenBank, all available sequences of male-specific Y-chromosome genes (sdY) in salmonid species were analyzed for specific oligonucleotide primer design. The PCR assay for rapid identification of males in rainbow trout *Onchorhynchus mykiss*, brown trout *Salmo trutta*, huchen *Hucho hucho* and grayling *Thymallus thymallus* was developed. The length of PCR products was in the range of 200–800 base pairs (bp). The specificity of the amplified fragments was tested by sequencing of PCR products. All PCR products corresponded to the areas of the Y chromosome where the sdY loci are located. The comparison of the amplified DNAs revealed high identity (95–99 %) between the sequences of the rainbow trout, the brown trout, the huchen, and the grayling. The highest identity rates were noted among one specific genus and the percentage of homology was approximately 99 % as shown for rainbow trout *O. mykiss*. **Conclusions**. The sex of the mentioned above fish species can be readily determined by the PCR assay which allows performing simple identification of "neomales" in the indirect feminization method via the hormonal sex reversal. The assay can be classified as express diagnostics, because the data analysis and the delivery of the generated results to the fish-farming site can be accomplished within a day.

Keywords: sex determination, PCR assay, male-specific genes, salmonids, indirect feminization, hormonal sex reversal.

INTRODUCTION

Monosex populations of fish are desirable in aquaculture for a variety of reasons. The male grows faster in some species and the female – in other species. In this case, the monosex culture of the faster-growing sex can increase the production and the sexual dimorphism in growth occurs in most cultured fish [1]. The males of some species of salmonids can become sexually mature early, at a sub-optimal market size. To alleviate this problem, all-female strains have been developed in some countries and now make up the bulk of production of those species worldwide [2, 3].

Salmonids possess an XY genetic sex determination system which is highly stable, allowing for pure populations of XX individuals for the production purposes. The females of salmonids grow faster than the males so the breeding of all-female populations is very beneficial for fish producers. All-female populations of salmonids can be obtained via the hormonal sex reversal by the indirect feminization method [4]. The principal step of this method is to identify and discard genotypic males, because only "neomales" (phenotypic males with XX genotype) must be used in the next crosses with native females to get 100 % all-female stock. The two types of males have been distinguished from one another as follows:

1) test-crossing each individual with regular females, and retaining those which yield only female progeny (which takes many years to accomplish);

2) using distinguishing characteristics (i.e. lack of a spermduct) that exist between XX and XY males (inducing this characteristic requires long-term dietary

treatments with androgen and is not always reliable);

3) the identification of hermaphrodite gonads, which is strongly indicative of an XX genetic background (but only occurs in a small proportion of sex-reversed fish and thus is also unreliable);

4) the use of Y-chromosomal DNA markers which can distinguish males of XX and XY genotype (which is very reliable), thus the conventional PCR can be used [5, 6].

The rainbow trout (*O. mykiss*), the brown trout (*S. trutta*), the huchen (*H. hucho*) and the grayling (*T. thymallus*) are the most popular species of salmonids in Ukraine. Due to their high food value and simplicity of breeding, these fishes are wide-spread and basic objects of fish-farming. Recent research with sex-determining sequences in 15 different species of salmonids revealed a highly conserved and male-specific Y-chromosome gene sdY [7]. Based on the wide conservation of sdY as a male-specific Y-chromosome gene, efficient and easy molecular sexing techniques can be developed. Therefore, the aims of our research were to identify the sdY locus in the mentioned above salmonid species and develop the PCR technique for rapid fish sex determination.

MATERIALS AND METHODS

DNA extraction. Forty fins from sex-maturated rainbow trout, brown trout, huchen and grayling (n = 5 of each sex and species) were independently homogenizated in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4). Genomic DNA was extracted using a 25:24:1 phenol:chloroform:isoamyl alcohol (IAA) mixture, followed by ethanol precipitation [8]. Briefly, the fin homogenates were incubated with proteinase K in the presence of lysis buffer (10 mM Tris-HCl, 0.1 M EDTA, 0.5 % (w/v) SDS, pH 8.0) for 2 h at 37 °C and then treated with phenol:chloroform:IAA mixture. In order to separate organic solvents and water-dissolved DNA, the mixture was centrifuged at 13,000 g for 5 min. A 0.1 volume of 3 M sodium acetate and 2.5 volumes of cold 95 % ethanol were added to the aqueous phase. The mixture was incubated for 1 h at 20 °C for DNA precipitation, followed by the centrifugation at 13,000 g. The supernatant was removed and the pellet was washed with cold 70 % ethanol and then air-dried. The nuclease-free water was added and resuspended for DNA dissolving. The DNA concentrations were determined by the spectrophotometer (APEL PD-303 UV, Japan) and the samples were stored at –20 °C until use.

Primers design. We selected four primer pairs in the sexually dimorphic Y-chromosome locus (sdY) for conventional PCR technique. For this purpose we used all the available nucleotide sequences of salmonids in NCBI (www.ncbi.nim.nih.gov). The selected oligonucleotides were tested for possible secondary structure and self-complementarity using Vector NTI 10 software (INVITROGEN, USA) and the on-line service BLAST (www.ncbi.nim.nih.gov/blast). All the oligonucleotides were synthesized commercially by *Metabion* (Germany).

*PCR assay***.** The reagents used for PCR amplification were obtained from *ThermoScientific* (USA). Each 25 µl of the reaction mixture (prepared on ice) contained the DNA sample, 12.5 µl of DreamTaq PCR Master Mix, 20 pmol of each oligonucleotide primer and distilled water up to total volume. A PeqStar (PEQLAB, Germany) thermocycler was used for PCR as follows, Cycle 1: 95 °C for 2 min; Cycles 2 to 30 : 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min; Cycle 31 : 72 °C for 5 min. The PCR mixture containing no DNA template was used as the control. After the PCR all products were transferred to 2.0 % agarose gel, electrophoresed, and the DNA was visualized by ethidium bromide staining.

Sequencing. Each fragment of the amplified DNA for different species was taken for sequencing. The Silica Bead DNA Extraction Kit (*ThermoScientific*, USA) was used for the extraction of DNA from agarose gel. The sequencing was performed on a 3130 Genetic Analyzer and analyzed using BLASTN, Vector NTI 10 and MEGA software version 5.2.

RESULTS AND DISCUSSION

All the available nucleotide sequences of sdY locus were analysed using BLASTN and MEGA software. The comparison of sdY sequences in salmonids revealed the high identity of 95–99 % within *Onchorhynchus*, *Salmo, Hucho, Thymallus, Salvelinus* genera and others (Fig. 1).

Our results showed that the selected oligonucleotide primers for sdY locus amplified the same products for all the tested salmonid species. The PCR products were 800 bp long for the rainbow trout, approximately 500 bp for the huchen, the fragment for the grayling was about 600 bp and the product in 200 bp was that of the brown trout (Fig. 2).

The cross amplification was performed in order to establish the specificity of primers. The specific primers for the rainbow trout and brown trout and also their

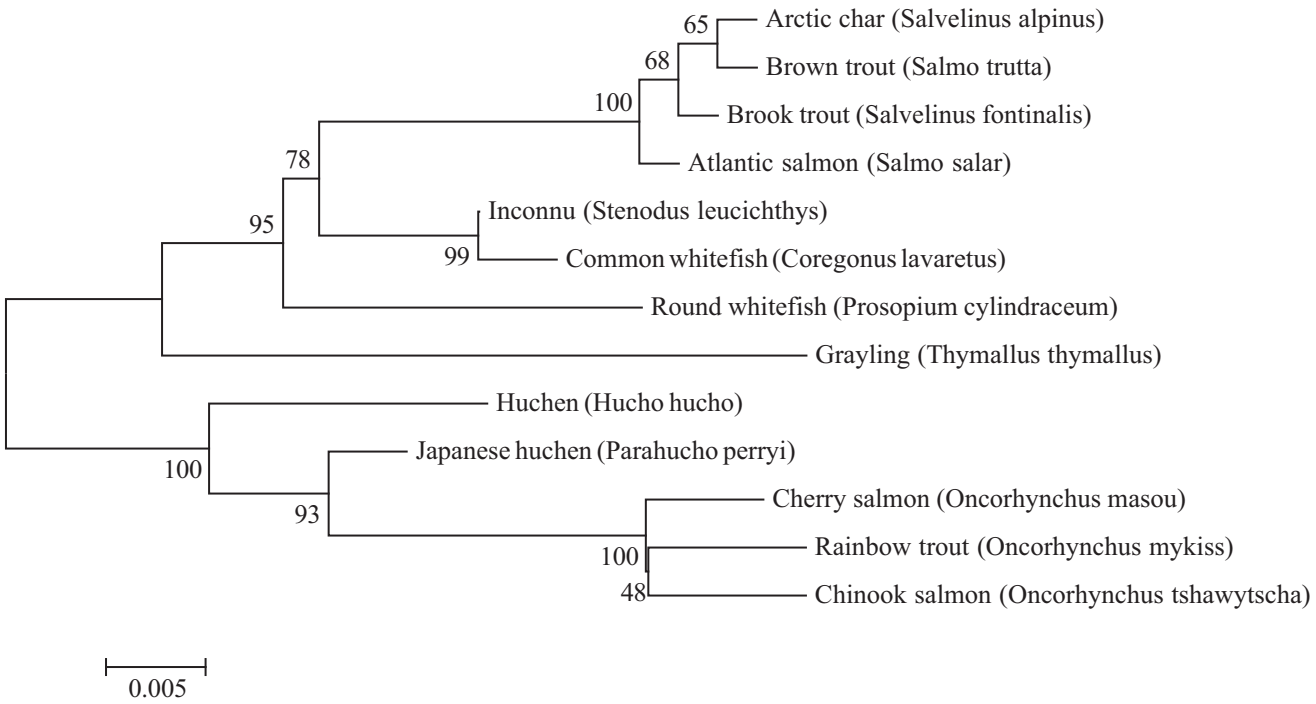

Fig. 1. The phylogenetic three of sdY locus of salmonid species, generated by means of a neighbor-joining algorithm in MEGA software version 5.2

DNA samples were used in simple PCR. It was shown that specific oligonucleotides for the brown trout did not amplify PCR products with DNA of the rainbow trout and vice versa there were no products in the reaction of the rainbow trout specific primers and the brown trout DNA (Fig. 3).

The specificity of the amplified fragments was tested by sequencing of PCR products. It was shown that the sequences of amplified fragments corresponded to the areas of the Y chromosome where the sdY loci were located. The comparison of the amplified sdY locus sequences of the investigated fish revealed high identity of 95–99 % between the samples of *O. mykiss*, *S. trutta, H. hucho* and *T. thymallus*. The highest identity rates were noted within certain genera and the percentage of homology was approximately 99 % as shown for rainbow trout *O. mykiss* in Fig. 4.

All the salmonid species investigated up to date have been characterized using the male heterogametic sex determination system. However, as these species do not share any Y-chromosome conserved synteny, there remains a debate on whether they share a common master sex-determining gene. Yano *et al*. traced the extent of conservation and evolution of the master sex-determining genes sdY in 15 different salmonid species [7]. Using MEGA software and BLASTN we showed that the sdY sequence is highly conserved in all salmonids and

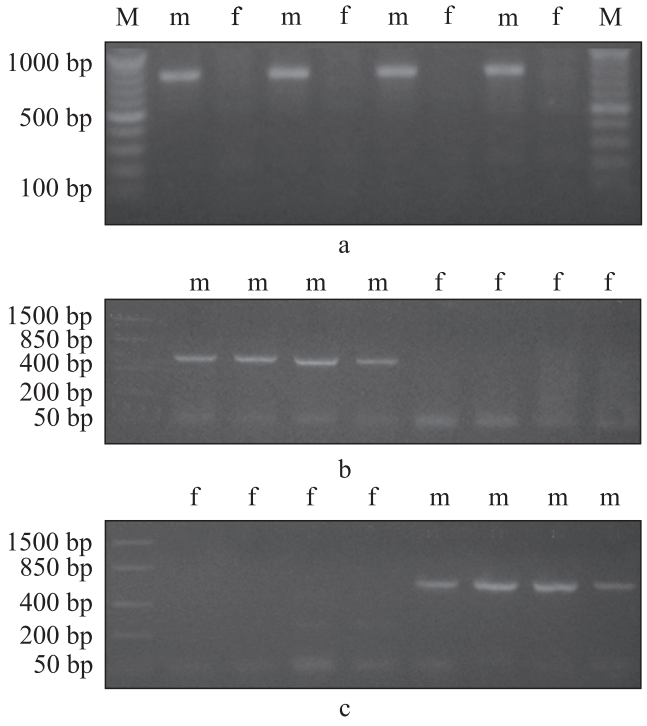

Fig. 2. The amplification of the rainbow trout (a), the huchen (b) and the grayling (c) genomic DNA by PCR, incorporating the selected primers: m – male, f – female, line M – Quick Load 100 bp DNA ladder (BioLabs, UK). The positive control of DNA (beta-actin gene) and no DNA template as a negative control were used in the amplification respectively (not indicated)

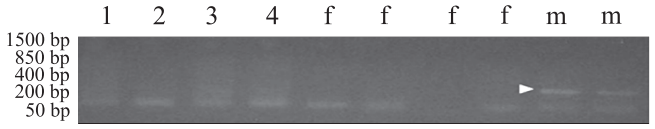

Fig. 3. The cross amplification of sdY locus fragments from the DNA samples of the rainbow and brown trout using species-specific oligonucleotide primers: 1, 2 and 3, 4 – males and females of rainbow trout *O. mykiss*; m – male (►) and f – female of brown trout *S. trutta*, line M – Quick Load 100 bp DNA ladder (BioLabs, UK).

that sdY is a male-specific Y-chromosome gene for the majority of these species. These findings demonstrate that most salmonids share a conserved sex-determining locus and also strongly suggest that sdY may be this conserved master sex-determining gene. Therefore, these data may be of great interest for further study of these economically and environmentally important fish species.

Thus, as a result of primer selection, the PCR methods for rapid diagnostics of fish sex in the rainbow trout, the huchen, the grayling, and the brown trout were developed. The discarding of genotypic males and selection of "neomales" are very important moments in the indirect feminization method via the hormonal sex reversal (EU Directive 96/22/CE, dated April 29, 1996). In order to get 100% all-female stock, only "neomales" must be selected for subsequent crossing with regular females.

Some salmonid species demonstrate moderate to high levels of early sexual maturation, at a sub-optimal market size. The development of all-female strains of these species could be a significant benefit for the aquaculture industry as it would allow preventing the losses arising from early maturation of males [9]. The precocious maturation of salmonid species (usually one year for males and two years for females) induces great variability in flesh quality, as well as high male mortality due to the *Saprolegnia* fungus and low rates of resistance to infectious hematopoietic necrosis virus (IHN) and to viral haemorrhagic septicaemia (VHS) [10]. Several authors have underlined the potential interest of farming immature all-female or sterile all-female salmonid populations to solve these problems and to achieve bigger sizes, more adapted for the fillet market and with a better feed conversion index [11]. The farming of sterile fish is also advocated by FAO to prevent genetic interaction from the escaped farmed genotypes with wild populations, in the interests of sustainable aquaculture [12].

In addition to the benefits of monosex strains derived from elimination of sexual maturity, all-female strains also allow for the enhanced roe production which can be of benefit if appropriate markets have been developed. Thus, for the huchen, the use of monosex female strains has allowed for a doubling in the roe production from the same number of production animals previously used in the mixed sex culture.

Consequently, based on the wide conservation of sdY as a male-specific Y-chromosome gene, we selected efficient primers for easy molecular sex selection techniques such as conventional PCR. Our results showed that selected oligonucleotide primers for the sdY locus amplified the same products for all the tested salmonid species. The PCR products were 800 bp long for the rainbow trout, 500 bp for the huchen, 600 bp for the grayling, and 200 bp for the brown trout. The sdY primers used for amplification yielded no products in

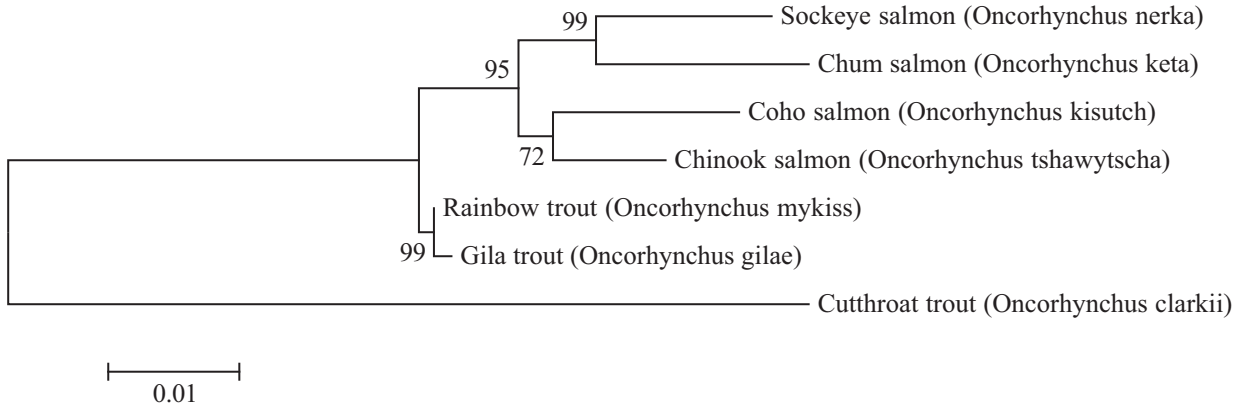

Fig. 4. The analysis of the amplified product sequences of sdY locus in rainbow trout *O. mykiss* (Ishhan fish farm, Chernivtsi region, Western Ukraine) and its comparison with the identical fragments from other members of *Onchorhynchus* genus. The tree was generated by means of a neighbor-joining algorithm in MEGA software version 5.2

genetic females, whereas single fragments were produced in the males of the tested species. The specificity of the amplified fragments was tested by sequencing of PCR products. It was shown that the sequences of the amplified fragments corresponded to the areas of the Y chromosome where the sdY loci were located. The comparison of the amplified DNA revealed high identity of 95–99 % between the sequences of *O. mykiss*, *S. trutta, H. hucho* and *T. thymallus*.

The investigations on the sex-linked DNA-markers will be continued in our future research due to its urgency for biotechnology and step-by-step transition to all-female strains of salmonid aquaculture in Ukraine.

CONCLUSIONS

The PCR diagnostics for sex determination in rainbow trout *O. mykiss*, brown trout *S. trutta*, huchen *H. hucho* and grayling *T. thymallus* were developed. The sex genotype can be readily determined by conventional PCR diagnostics which can be performed using crude tissue (fins) or blood as source of DNA. The assay can be classified as express diagnostics, because the data analysis and the delivery of the generated results to the fish-farming site can be accomplished within a day. The application of simple PCR-based diagnostics allows distinguishing regular XY males from masculinized XX males, facilitating the development and maintenance of monosex, all-female strains.

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Системи визначення статі у лососевих видів риб України методом полімеразної ланцюгової реакції

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Мета. Проаналізувати послідовності ДНК статевоспецифічних маркерів лососевих видів риб та розробити експрес-діагностику статі у райдужної форелі *O. mykiss*, струмкової форелі *S. trutta*, дунайського лосося *H. hucho* та харіуса європейського *T. thymallus*. **Методи.** Аналіз ДНК-послідовностей, філогенетичний аналіз, підбір специфічних олігонуклеотидних праймерів, виділення ДНК, полімеразна ланцюгова реакції (ПЛР), реакція секвенування. **Результати.** За даними аналізу статевоспецифічних локусів (sdY) лососевих видів риб підібрано олігонуклеотидні праймери для експрес-діагностики самців у райдужної форелі, струмкової форелі, дунайського лосося та харіуса європейського. Розміри продуктів ампліфікації становили від 200 до 800 пар нуклеотидів. Специфічність реакцій ампліфікації перевірено аналізом нуклеотидного складу ампліконів. Усі продукти ПЛР відповідали ділянці Y хромосоми, де знаходяться локуси sdY. Порівняльне вивчення нуклеотидних послідовностей ампліфікованих фрагментів та локусу sdY райдужної форелі, струмкової форелі, дунайського лосося та харіуса європейського засвідчило високий ступінь ідентичності, який становить 95–99 %. Максимальну ідентичність ДНК-послідовностей – 99 % – спостерігали при дослідженні сіквенсів одного роду, наприклад *Onchorhynchus*. **Висновки.** За допомогою підібраних специфічних олігонуклеотидних праймерів стать райдужної форелі, струмкової форелі, дунайського лосося та харіуса європейського може бути визначена методом традиційного ПЛР. Це дозволяє ідентифікувати самців-реверсантів під час процесу гормональної реверсії статі у риб. Даний метод належить до експрес-діагностики, оскільки аналіз даних і представлення результатів до рибного господарства можливе упродовж однієї доби.

Ключові слова: ідентифікація статі, ПЛР, ДНК-маркери, лососеві риби України, гормональна реверсія.

Системы определения пола у лососевых видов рыб Украины методом полимеразной цепной реакции

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Цель. Проанализировать последовательности ДНК полоспецифических маркеров лососевых видов рыб и разработать экспресс-диагностику пола у радужной форели *O. mykiss*, ручьевой форели *S. trutta*, дунайского лосося *H. hucho* и хариуса европейского *T. thymallus*. **Методы.** Анализ ДНК-последовательностей, филогенетический анализ, подбор специфических олигонуклеотидных праймеров, выделение ДНК, полимеразная цепная реакции (ПЦР), реакция секвенирования. **Результаты.** По данным анализа полоспецифических локусов (sdY) лососевых видов рыб подобраны олигонуклеотидные праймеры для экспресс-диагностики самцов у радужной форели, ручьевой форели, дунайского лосося и хариуса европейского. Размеры продуктов амплификации составляли от 200 до 800 пар нуклеотидов. Специфичность реакция амплификации проверена анализом нуклеотидного состава ампликонов. Все продукты ПЦР отвечали участку Y хромосомы, где находятся локусы sdY. Сравнительное изучение нуклеотидных

последовательностей амплифицированных фрагментов и локусов sdY радужной форели, ручьевой форели, дунайского лосося и хариуса европейского выявило высокую степень идентичности, составляющую 95−99 %. Максимальную идентичность ДНК-последовательностей − 99 % – наблюдали при исследовании сиквенсов одного рода, например *Onchorhynchus*. **Выводы.** С помощью подобранных специфических олигонуклеотидных праймеров пол радужной форели, ручьевой форели, дунайского лосося и хариуса европейского может быть определен методом традиционной ПЦР. Это позволяет идентифицировать самцов-реверсантов во время процесса гормональной реверсии пола у рыб. Данный метод принадлежит к экспресс-диагностике, поскольку анализ данных и возвращение результатов в рыбное хозяйство осуществляются на протяжении одних суток.

Ключевые слова: идентификация пола, ПЦР, маркеры ДНК, лососевые рыбы Украины, гормональная реверсия.

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