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(54) Titre : DETERMINATION DU SEXE PAR PCR EN TEMPS REEL CHEZ LE SAUMON COHO
(54) Title: SEX DISCERNMENT BY REAL-TIME PCR IN COHO SALMON

(57) Abrégé/Abstract:
A sex discernment method that uses real-time polymerase chain reaction (PCR) to discern the sex of Coho salmon (Oncorhynchus kisutch) comprises the following steps: extracting and amplifying Coho salmon DNA by PCR, using the fluorescent probe pCsex1 (FAM) 5'-CCTGTGTATACTTGGGAGCGGTAAGCC-3' and a mixture of primers, CSex2s: 5'-CATTATATTTGGCTGAAGAGTCAGTG-3' and CSex2r: 5'-GGAACAGATGGAGCGGTCTTC-3'; identifying the presence of the sequence CATTATATTTGGCTGAAGAGTCAGTGTTCTGCTTGTATTAATATAATATAGAGCTTGAATTTAGACTTGAATTCTCACAGTTG GAGGTCAGTTTAGTTATGAATTAAACCTGTGTATACTTGGGAGCGGTAAGCCATGTGCCCATTTACAAAGTCTGACCAGAG AAGACCGCTCCATCTGTTCC, as a fluorescence signal, the presence of the fluorescence signal indicating male Coho salmon and the absence of the fluorescence signal indicating female Coho salmon. The mixture of primers and the kit comprising the mixture of primers and the fluorescent probe are also disclosed.

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(54) **Title**: SEX DISCERNMENT BY REAL-TIME PCR IN COHO SALMON

(54) **Título** : DETERMINACIÓN DEL SEXO MEDIANTE PCR TIEMPO REAL EN SALMÓN COHO

(57) **Abstract**: A sex discernment method that uses real-time polymerase chain reaction (PCR) to discern the sex of Coho salmon (*Oncorhynchus kisutch*) comprises the following steps: extracting and amplifying Coho salmon DNA by PCR, using the fluorescent probe pCsex1 (FAM) 5'-CCTGTGTATACTTGGGAGCGGTAAGCC-3' and a mixture of primers, CSex2s: 5'-CATTATATTTGGCTGAAGAGTCAGTG-3' and CSex2r: 5'-GGAACAGATGGAGCGGTCTTC-3'; identifying the presence of the sequence
CATTATATTTGGCTGAAGAGTCAGTGTTCTGCTTGTATTAATATAATATAGAGCTTGAATTTAGACTTGAATTCTCACAG
TTGGAGGTCAGTTTAGTTATGAATTAAACCTGTGTATACTTGGGAGCGGTAAGCCATGTGCCCATTTACAAAGTCTGACC
AGAGAAGACCGCTCCATCTGTTCC, as a fluorescence signal, the presence of the fluorescence signal indicating male Coho salmon and the absence of the fluorescence signal indicating female Coho salmon. The mixture of primers and the kit comprising the mixture of primers and the fluorescent probe are also disclosed.

(57) **Resumen**: Un método para determinar el sexo mediante el uso de la reacción de polimerasa en cadena (PCR) tiempo real en salmón coho (*Oncorhynchus kisutch*) que comprende los siguientes pasos: extraer y amplificar ADN de salmón coho mediante PCR, usando la sonda fluorescente pCsex1 (FAM) 5'-CCTGTGTATACTTGGGAGCGGTAAGCC-3' y una mezcla de partidores CSex2s: 5 '-CATTATATTTGGCTGAAGAGTCAGTG-3 ' y CSex2r: 5'-GGAACAGATGGAGCGGTCTTC-3'; identificar la presencia de la secuencia
CATTATATTTGGCTGAAGAGTCAGTGTTCTGCTTGTATTAATATAATATAGAGCTTGAATTTAGACTTGAATTCTCACAG
TTGGAGGTCAGTTTAGTTATGAATTAAACCTGTGTATACTTGGGAGCGGTAAGCCATGTGCCCATTTACAAAGTCTGACC
AGAGAAGACCGCTCCATCTGTTCC, como una señal de fluorescencia, donde la presencia de dicha señal de fluorescencia, determina sexo masculino en el salmón coho, y a la ausencia de la dicha señal de fluorescencia, determina sexo femenino en el salmón coho. La mezcla de partidores y el kit que comprende la mezcla de partidores y la sonda fluorescente.

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SEX DISCERNMENT BY REAL-TIME PCR IN COHO SALMON

Field of the Invention

The present invention relates to a method for discerning sex by the use of real-time polymerase chain reaction (PCR) in *coho* salmon *(Oncorhynchus kisutch)*, related primers and kit.

State of the Art

The real-time polymerase chain reaction (PCR) technique consists of the *in vitro* amplification of a specific sequence of interest, whose amplification generates a fluorescent signal detectable by the equipment. In function of the signal's behaviour, we can establish the abundance of the amplified sequence. Therefore, we can have two results with this technique, the first one consists of establishing the presence or absence of a sequence of interest in a sample being analyzed, and secondly, we can establish a kind of relative quantification of this sequence in the sample.

In several species, wherein sex determination is given by heterogametic chromosomes, in which in one of the chromosomes there exists a gene containing the information for determining sex, in the case of the human species this gene is designated as *sry*, which is located in chromosome Y. In salmonid species, it is also known that sex determination is given by heterogametic chromosomes. However, a *Sry*-type gene as in humans has not been found to this date.

In the Crossing Programs for *coho* salmon, in culture, it is only required to have a proportion of 1/3 males relative to females for the generation of a sufficient number of reproductors.

One object of the present invention is to provide a method for discerning sex in *coho* salmon which would allow to confirm the existence of a sufficient amount of males for crossings, rather than an excess or reduction of same.

In addition, the method of the present invention allows to evaluate "Neomale" production systems in *coho* salmon production systems. Neomale fish are females which through various methodologies revert to a male phenotype. However, at the genetic sequence level, they do not exhibit changes, these only occur at the phenotypic level. Therefore, this real-time PCR methodology may be applied to evaluate the efficiency of Neomale production in a Genetic Program in *coho* salmon rearing.

Brief Description of the Invention

Based on bioinformatic analyses, a putative genomic region was identified which would be present only in males of the *coho* salmon species, which for convenience we will designate as "Cohoseq" in the text, and comprises the sequence

CATTATATTTGGCTGAAGAGTCAGTGTTCTGCTTGTATTAATATAATATAGAGCTTAA

TTTAGACTTGAATTCTCACAGTTGGAGGTCAGTTTAGTTATGAATTAAACCTGTGTAT

ACTTGGGAGCGGTAAGCCATGTGCCCATTTACAAAGTCTGACCAGAGAAGACCGCTC

CATCTGTTCC; to evaluate the presence of this sequence in a biological sample, a real-time PCR application was developed which allows to evaluate the presence of this sequence in the sample. Up to date, there does not exist in the literature any methodology for conducting sex determination in *coho* salmon which incorporates the use of real-time PCR.

The present invention relates to a method for discerning sex through the use of real-time polymerase chain reaction (PCR) in *coho* salmon (*Oncorhynchus kisutch*), which comprises the following steps:

a) extracting deoxyribonucleic acid (DNA) from *coho* salmon either from biological or solid or semisolid matrices, such as muscle, fin, gill or the like, or fluids, such as total blood, plasma and serum;

optionally storing the DNA eluate obtained in step (a) at 4°C;

b) amplifying the DNA eluate from step (b) by the use of polymerase chain reaction (PCR), in real-time format, using the fluorescent probe pCsex1 which corresponds to the sequence (FAM) 5´-CCTGTGTATACTTGGGAGCGGTAAGCC-3´ and a mixture of primers comprising

the CSex2s primer: 5′-CATTATATTTGGCTGAAGAGTCAGTG-3′ and the CSex2r primer: 5′-GGAACAGATGGAGCGGTCTTC-3′;

c) identifying the presence of the sequence CATTATATTTGGCTGAAGAGTCAGTGTTCTGCTTGTATTAATATAATATAGAGCTTGA ATTTAGACTTGAATTCTCACAGTTGGAGGTCAGTTTAGTTATGAATTAAACCTGTGTA TACTTGGGAGCGGTAAGCCATGTGCCCATTTACAAAGTCTGACCAGAGAAGACCGCT CCATCTGTTCC, as a fluorescence signal, in the amplification resulting from step (c), assigning to the presence of said fluorescence signal the determination of male sex in *coho* salmon, whose DNA was amplified, and to the absence of said fluorescence signal, the determination of female sex in *coho* salmon, whose DNA was amplified.

The present invention also relates to a mixture of primers for discerning sex through the use of real-time polymerase chain reaction (PCR) in *coho* salmon (*Oncorhynchus kisutch*), which comprises the synthetic sequences

CSex2s: 5′-CATTATATTTGGCTGAAGAGTCAGTG-3′ and

CSex2r: 5′-GGAACAGATGGAGCGGTCTTC-3′.

In addition, the invention comprises a kit for discerning sex through the use of real-time polymerase chain reaction (PCR) in *coho* salmon (*Oncorhynchus kisutch*), which comprises:

a) a mixture of primers comprising the synthetic sequences

CSex2s: 5′-CATTATATTTGGCTGAAGAGTCAGTG-3′ and

CSex2r: 5′-GGAACAGATGGAGCGGTCTTC-3′;

b) a fluorescent probe comprising the sequence:

pCsex1 (FAM) 5′-CCTGTGTATACTTGGGAGCGGTAAGCC-3′(BHQ).

Brief Description of the Figures

Figure 1: Amplification curve by real-time PCR in sex determination of *coho* salmon, the curve represents the presence of genetic sequence only in males.

Example, methodology

The extraction of genetic material can be performed from multiple sources. In the case of using solid or semisolid biological matrices, such as muscle, fin, gill, etc., the sample is placed inside a bag and is macerated with a rubber hammer. Subsequently, phosphate buffered saline (PBS) buffer is added enough to reach a suitable turbidity (equivalent to No. 5 of the McFarland scale). Samples corresponding to fluids (total blood, plasma and serum) are directly treated. Subsequently, the DNA (deoxyribonucleic acid) extraction is performed, for example, with a

commercial kit. For this example, the protocol of the commercial kit QIAamp®DNA Blood Mini Kit will be used (Qiagen; catalog No. 51104X), wherein the description of the methodology will mention solutions whose abbreviations correspond to the original ones disclosed in the commercial kit (for example, AL buffer). To begin with the DNA extraction, 200 ml of sample (homogenate or fluid, whichever the case may be) are taken and introduced into an Eppendorf tube which has not been previously used, 20 ul Proteinase K (Invitrogen) are added and it is gently homogenized to be incubated during 30 minutes at 60°C. Subsequently, 200 ul of AL buffer are added to the sample in the tube. Homogenize by vortexing for 15 seconds and incubate at 56°C for 12 min. Briefly centrifuge at low revolution speed in order to avoid leaving drops (3000 rpm for 10 sec) on the edges. Add 200 ul of absolute ethanol (96-100%) and homogenize by vortexing for 15 seconds. Carefully add the homogenate to a column and centrifuge at 10000 x g for 1 min. Subsequently, change the collection tube to another collection tube which has not been previously used. Add 500 ul of AW1 buffer, incubate for 1 min in the column and centrifuge at 10000 x g for 1 min. Change the collection tube to another collection tube which has not been previously used. Add 500 ul of AW2 buffer, incubate for 1 min in the column and centrifuge at maximum speed for 3 min. Place the column in another collection tube which has not been previously used and add 50 ul of molecular biology grade water, and incubate at room temperature for 5 min. Finally, centrifuge at maximum speed for 1 min and store the eluate (DNA; deoxyribonucleic acid) at 4°C before use thereof.

In order to carry out the amplification phase of the genetic material, PCR in real-time format is used. For this part of the assay, for example, a commercial kit (Express qPCR Supermix Universal Kit (Invitrogen)) is used; for this purpose, a number of reactions based on the number of samples to be analyzed will be prepared. For example, for preparing 10 amplification reactions, prepare the microplates corresponding to this number of reactions and prepare the master mix as described in the kit; the following table shows the volumes of each component for preparing the master mix:

Master Mix	10X (ul)
Express qPCR SuperMix with Premixed ROX 10	100
Fluorescent probe pCsex1 [(1)] (0.3 uM)	10
Mixture of primers [(2)] (0.9 uM)	10
Nuclease-free water	70

(1) pCsex1 (FAM, 5'-CCTGTGTATACTTGGGAGCGGTAAGCC-3' (BHQ).

FAM: 6-carboxy-fluorescein (fluorophore)

BHQ: Black Hole Quencher (Quencher; fluorescent silencer)

Nuclease-free water: water that is free of nucleases which are enzymes that degrade genetic material.

(2) Primer sequences:

CSex2s: 5'-CATTATATTTGGCTGAAGAGTCAGTG-3' and

CSex2r: 5'-GGAACAGATGGAGCGGTCTTC-3'.

Subsequently, 19 ul of the master mix are placed in each of the microtubes and 1 ul of the previously extracted DNA sample is introduced into each one. Subsequently, place the tubes in a real-time thermocycler with the following thermal profile or schedule:

1 cycle: 95°C, 20 seconds

40 cycles: 95°C, 10 seconds

55°C, 15 sec*, fluorescence reading

1 cycle: 25°C, 30 seconds

At the end of the amplification schedule, the results are interpreted as detailed below.

In each microtube in which a real-time PCR reaction was conducted for discerning sex in a *coho* salmon sample, two events may occur. First, that a fluorescence signal is generated, which is interpreted as the presence of the Cohoseq sequence, which in turn is interpreted as male sex of

the salmonid since the female should not have this region. Second, that a fluorescence signal is not generated, which is interpreted as female sex since the Cohoseq sequence is not found, and therefore, there is no substrate for the generation of a signal. Figure 1 shows an analysis of some samples, it can be observed that there are several amplification events. The amplifying curves correspond to samples that were identified as male *coho* salmon, while the samples that did not amplify correspond to samples that were identified as female *coho* salmon.

In parallel, 60 *coho* salmon samples were analyzed, 30 female and 30 male, fish whose sex was previously established by visual inspection since they were adult specimens. Then, the samples were submitted to a real-time PCR analysis, obtaining 100% agreement of the results obtained with the prior information related to the sex of the samples, which was previously established by visual inspection.

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CLAIMS

1. A method for discerning sex through the use of real-time polymerase chain reaction (PCR) in *coho* salmon (*Oncorhynchus kisutch*) CHARACTERIZED in that it comprises the following steps:

a) extracting deoxyribonucleic acid (DNA) from *coho* salmon either from biological or solid or semisolid matrices, such as muscle, fin, gill or the like, or fluids, such as total blood, plasma and serum;

b) amplifying the DNA eluate from step (b) by the use of polymerase chain reaction (PCR), in real-time format, using the fluorescent probe pCsex1 which corresponds to the sequence (FAM) 5′-CCTGTGTATACTTGGGAGCGGTAAGCC-3′ and a mixture of primers comprising the CSex2s primer: 5′-CATTATATTTGGCTGAAGAGTCAGTG-3′ and the CSex2r primer: 5′-GGAACAGATGGAGCGGTCTTC-3′;

c) identifying the presence of the sequence CATTATATTTGGCTGAAGAGTCAGTGTTCTGCTTGTATTAATATAATATAGAGCTTGA ATTTAGACTTGAATTCTCACAGTTGGAGGTCAGTTTAGTTATGAATTAAACCTGTGTA TACTTGGGAGCGGTAAGCCATGTGCCCATTTACAAAGTCTGACCAGAGAAGACCGCT CCATCTGTTCC, as a fluorescence signal, in the amplification resulting from step (c), assigning

to the presence of said fluorescence signal the determination of male sex in *coho* salmon, whose DNA was amplified, and to the absence of said fluorescence signal, the determination of female sex in *coho* salmon, whose DNA was amplified.

2. Mixture of primers for discerning sex through the use of real-time polymerase chain reaction (PCR) in *coho* salmon (*Oncorhynchus kisutch*), CHARACTERIZED in that it comprises the synthetic sequences

CSex2s: 5′-CATTATATTTGGCTGAAGAGTCAGTG-3′ and

CSex2r: 5′-GGAACAGATGGAGCGGTCTTC-3′.

3. Kit for discerning sex through the use of real-time polymerase chain reaction (PCR) in *coho* salmon (*Oncorhynchus kisutch*), CHARACTERIZED in that it comprises:

a) a mixture of primers comprising the synthetic sequences

CSex2s: 5′-CATTATATTTGGCTGAAGAGTCAGTG-3′ and

CSex2r: 5′-GGAACAGATGGAGCGGTCTTC-3′;

b) a fluorescent probe comprising the sequence:

pCsex1 (FAM) 5′-CCTGTGTATACTTGGGAGCGGTAAGCC-3′(BHQ).

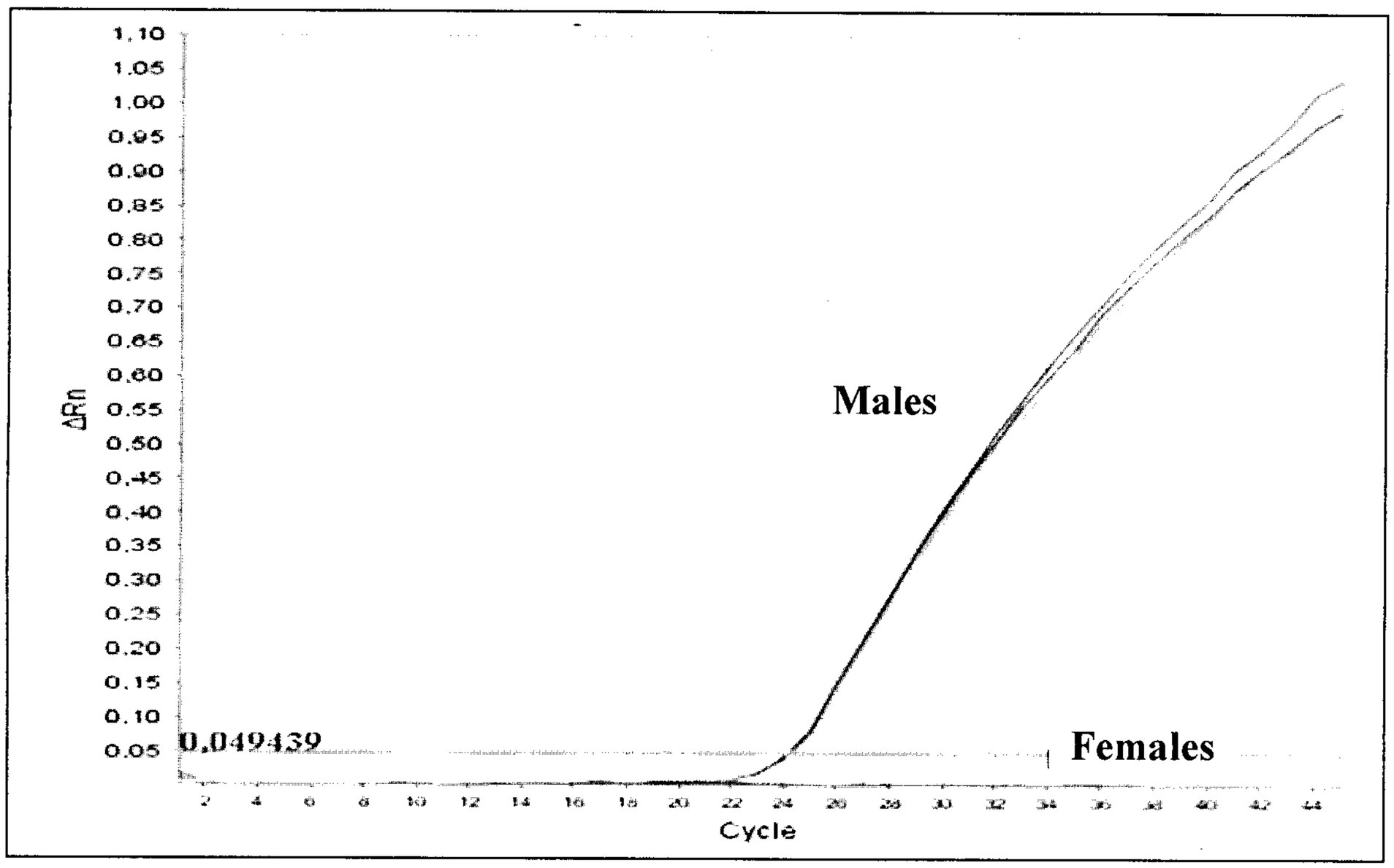

Figure 1