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SHORT COMMUNICATION

Rapid sex identification of Atlantic salmon (*Salmo salar* L.) by real-time PCR

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Morphological sexing of Atlantic salmon *Salmo salar* L. has proven to be difficult in immature stages as well as in adults. Non-lethal sex identification has therefore been a crucial need.

In 2013, the male-specific master sex-determining gene *sdY* (*sexually dimorphic on the Y chromosome*) was found to be conserved in nearly all salmonid species (Yano, Nicol, Jouanno, Quillet, Fostier, Guyomard & Guiguen 2013). A fragment of the first exon of the *sdY* gene was amplified and the polymerase chain reaction (PCR) products were revealed using agarose-gel electrophoresis.

Genetic sex determination at an early stage may be a valuable tool for the aquaculture industry due to several reasons. The surplus males can be terminated at an early stage consequently reducing the cost associated with tank space and feed. This opens up the possibility of increasing the ratio between females and males and thus increasing the production of salmon roe without increasing the space needed. Females are also preferred for the production of salmon fillets due to the potential early sexual development in males, which gives rise to poorer flesh quality.

Although, traditional DNA extraction methods and DNA revealing techniques, such as gel electrophoresis, allow accurate sex identification without terminal sampling, they are laborious and time-consuming. Therefore, the developments of fast cost-efficient methods suitable for sexing several thousand individuals are needed.

Quéméré, Perrier, Besnard, Evanno, Baglinière, Guiguen and Launey (2014) published a method based on fluorescent-labelled primers where the PCR products were run on an ABI Prism3130xl automated sequencer. The presence or absence of a 180–200-bp peak corresponded to male and female respectively. The genomic DNA was extracted from scales using the Chelex method (Estoup, Largiader, Perrot & Chourrout 1996).

Anglès d Auriac, Urke and Kristensen (2014) also proposed a method where the real-time duplex PCR was performed using a Bio-Rad CFX96. The melt curve analysis was used to score the presence of the amplification of the *sdY* male-specific marker as well as the 18S housekeeping gene. Although this analysis is rapid, it relies on traditional DNA extraction which is time-consuming.

Rud, Maistrenko and Buchatskii (2015) recently developed a method for sex identification in rainbow trout (*Oncorhynchus mykiss*). They used a laborious DNA extraction method, conventional PCR and gel electrophoresis.

This study presents an alternative to these previously published methods, using a simple pre-treatment with boiling water followed by a real-time PCR where a fluorescent probe was used to detect the *sdY* amplicon. The method was developed to be straightforward and compatible with large-scale production of salmon roe.

Fin samples were collected at the Aquaculture Research Station of the Faroes (Skopun, Faroe Islands) and stored in 96-well plates at −18°C. All individuals (below 300 g) were tagged with PIT-tags following the manufacturer's recommendations (Biomark, Boise, ID, USA) in order to identify the

individuals after the samples were analysed. The fish used in this study were sedated using Finquel (Tricaine mesylate) (Scan Aqua AS, Norway) during tagging and sample collection. Finquel was also used to euthanize the fish. The fish were kept in 234 m^3 tanks at Aquaculture Research Station of the Faroes.

The frozen samples were transported to the research park iNOVA (Tórshavn, Faroe Islands) where they were analysed. The pre-treatment consisted of sample incubation in 100 μL boiling water for 15 min. Subsequently, the samples were centrifuged at 2228 *g* (Sigma 4-15; Buch & Holm, Herlev, Denmark) for 5 min at room temperature. DNA concentration and 260 nm/280 nm ratio was measured for 92 random samples using a BioPhotometer plus (Eppendorf, Hørsholm, Denmark). The samples were diluted 50 times in Milli-Q water prior to real-time PCR. No commercial kit or chemicals were used prior to real-time PCR.

A short fragment (93 bp) within the 4th exon of the *sdY* gene (GeneBank association number: JF826020) was amplified using a QuantiTect PCR kit (Qiagen, Copenhagen, Denmark), a VIC® fluorescent probe and previously published primers (Eisbrenner, Botwright, Cook, Davidson, Dominik, Elliott, Henshall, Jones, Kube & Lubieniecki 2013) (DNA-Technology, Risskov, Denmark). The primer sequences used were: forward: 5′-AGTTGGAACGCTTCAGCAGAGCAGATGG-3′ and reverse: 5′-GGACAAGACTCATCACTCAGTGCACCAATCT-3′. The VIC® fluorescent probe (5′-CAACCGCAAATTGGGTTCAGCC-3′) which binds to the *sdY* amplicon was designed using the GenScript Real-time PCR (TaqMan) Primer Design online tool (https://www.genscript.com/ssl-bin/app/primer). The probe was a TaqMan probe with a 5′ VIC dye and a 3′ MGBNFFQ quencher (Life Technology, Nærum, Denmark).

All PCR amplifications were performed in a total volume of 10 μL with 2 μL of the diluted supernatant and a final concentration of 0.4 μM for the two primers and 0.25 μM for the probe. To minimize the cost of this analysis, only one PCR reaction was performed per individual. All pipetting was performed by a Biomek 3000 Robot (Beckman Coulter, Miami, FL, USA) in 96-well plates (Life Technology).

The one-step PCR amplifications were conducted using the following conditions: a denaturing step for 15 min at 95°C, followed by 40 cycles of 94°C for 15 s and 60°C for 60 s. Polymerase chain reactions were run on a StepOnePlus™ Real-Time PCR System (Life Technology).

An individual was considered a male if the *sdY* fragment was amplified while the female sex was assigned when no *sdY* amplification was observed. The presence or absence of the amplicon was identified by the StepOne™ Software (Life Technology). StepOnePlus™ Real-Time PCR System utilizes a long-life LED-based optical system that records fluorescence from the designed probe labelled with VIC® dye.

The method was developed in the laboratory, where 20 individuals were sampled, terminated and their gonads examined. This procedure was repeated 10 times with small alterations to optimize the method. The sex ratio was found to be roughly 50:50.

The method was tested on 2583 individuals in the Atlantic salmon roe production line at the Aquaculture Research Station of the Faroes. A

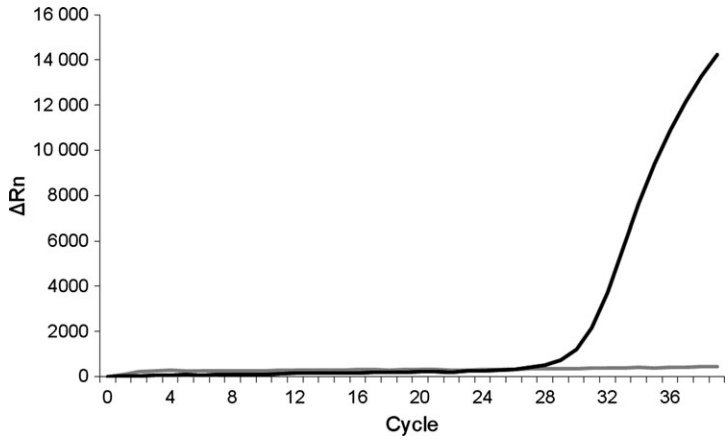

Figure 1 A typical *sdY* amplification plot with one *sdY*-positive (black) and one *sdY*-negative (gray) individual. Baseline-corrected normalized reporter (ΔRn) plotted against cycles.

total of 1257 (48.7%) of the tested individuals were positive for the *sdY* gene and when their gonads were examined only one was a female. This false positive result was most likely due to human error.

The expected sex ratio of 50:50 suggested that there might have been some false negative results. However, the false negative results were not evaluated because the *sdY*-negative individuals were not terminated as they were a part of a commercial salmon roe production line. Morphological sexing was not applied to identify the potential false negative individuals because it has proven to be very difficult in immature stages. The tested individuals in this study weighed below 300 g. A positive control would have minimized the impact of insufficient DNA quality and quantity causing false negative results. Nevertheless, no internal control gene was analysed due to added cost.

Figure 1 shows a typical amplification plot of an *sdY*-positive and an *sdY*-negative individual. The Ct values for the 1256 males were found to be between 28 and 34 cycles. The average DNA concentration was 444 ± 116 μg mL^{-1} (average ± SD; $n = 92$) and the average 260 nm/280 nm ratio was 1.2 ± 0.1 (average ± SD; $n = 92$). The low 260 nm/280 nm ratio indicates that the samples do not contain pure DNA. This is most likely due to the simple pre-treatment used in this study. However, the quantity and quality of the DNA was sufficient for the real-time PCR analysis used in this study to identify the majority of the males (48.7% out of the expected 50%).

In summary, this method was based on a simple tissue pre-treatment with boiling water for 15 min followed by real-time PCR and provided a straightforward and reliable sex identification of several hundred individuals. The differences between this method and previously published methods were the simple pre-treatment and the probe, which was used for specific *sdY* detection. This simple protocol could be useful for decreasing the number of surplus males in any commercial production of salmon roe and substituting males with females and thus increasing the production of salmon roe. Furthermore, we believe that this simple DNA preparation method may also be suitable for detecting other target genes and be applied to other species.

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Conflicts of interest

The authors have no conflict of interest to declare.

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