# Identification of a dominant SCAR marker associated with colour traits in Coho salmon (*Oncorhynchus kisutch*)

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**Abstract**

Flesh colour in salmonids is a very important commercial trait that shows additive genetic variance with significant environmental influence. Selective breeding for this trait is difficult, since phenotypic evaluation requires individuals to be sacrificed for scoring. We have identified a RAPD marker that co-segregates with colour traits scored as breeding values based on visual evaluations of flesh colour in Coho salmon. The experimental approach included RAPD screening with 440 primers and selective DNA pooling. Individuals used to make pools were selected according to their high and low breeding values as predicted using a DFREML algorithm. A single RAPD polymorphism segregating for flesh colour was used to derive a molecular single locus SCAR marker (*Oki*206, GeneBank accession AY661427) associated with muscle colour traits ($\chi^2=21.208$, $P<0.0001$) that has potential for use in marker assisted selection.

*Keywords:* Coho salmon; *Oncorhynchus kisutch*; Flesh colour; Breeding value; MAS; SCAR

## 1. Introduction

Red-orange flesh colour is an essential quality of farmed salmonid fish, which determines its market acceptance and price (Sigurgisladottir et al., 1997). Muscle colouration in salmonids is a complex trait and cannot be deduced from external morphology, which has prevented selection using traditional quantitative genetic methods. This problem has been partially solved in breeding programs by using scores from full sibs for breeding value predictions and selection (Gjedrem, 2000).

However, flesh colour is a good candidate trait to be improved more efficiently with marker assisted selection (MAS). The main limitations of quantitative genetic selection to improve a complex trait, such as muscle colour in salmonid fish, have been summarized by Dekkers and Hospital, 2002. Flesh colour has medium to low heritability in the main commercial species of salmon, fluctuating between 0.16 for

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Atlantic salmon (Gjerde and Gjedrem, 1984; Rye and Gjerde, 1996) and 0.28 for rainbow trout (Gjerde and Schaeffer, 1989; Kause et al., 2002). The phenotype is difficult to measure; there are two alternative methods to evaluate colour: a visual method using cards with standardized colours (Gjerde and Gjedrem, 1984) and an instrumental method using photocolourimetric analysis (Skrede and Storebakken, 1986).

Salmon has a long generation interval, 2–4 years depending upon the species and cultivation conditions (Stickney, 1991). Considering that this trait is measured in adult fish and that the individual needs to be sacrificed to score its muscle colour, thus losing it as a possible breeder, it would be extremely useful to have molecular markers to discriminate fish according to their ability to express this trait.

In this paper we used an approach to identify RAPD markers (Welsh and McClelland, 1990; Williams et al., 1990) associated with the genetic determinants of flesh colour in Coho salmon (*Oncorhynchus kisutch*). RAPD markers are anonymous segments of genomic DNA randomly PCR-amplified with an identical pair 10 oligonucleotide primers. Polymorphisms detected in a RAPD assay occur due to base substitutions at the annealing site or to indels in the region between primers binding sites (Liu and Cordes, 2004). The experimental method combined the use of predicted breeding values (PBV) in a strategy of selective DNA pooling (*sensu* Darvasi and Soller, 1992, 1994). Finally, we converted the RAPD marker that showed a significant association with colour traits into a dominant single locus SCAR marker (Paran and Michelmore, 1993), which has potential applications in breeding programs for genetic improvement. This approach is novel in the use of the PBV for colour instead of the individual phenotype to associate it with a particular DNA marker, as is usually done. The phenotype is used assuming that phenotypic differences between individuals are good predictors of genotypic differences, which is indeed true for quantitative traits of high heritabilities. For a low heritability trait such as flesh colour, our proposition is that if we are searching for DNA marker associated with this trait it will be more efficient to use a PVB as a BLUP estimate, instead of the individual phenotype.

## 2. Material and methods

### 2.1. Experimental population

A sample of 971 Coho salmon (498 males and 473 females) was obtained from the Genetic Improvement Program at the Dr. Shiraishi Hatchery in Coyhaique, XI Region of Chile. In this program, salmon with the highest harvest weight breeding values are selected as breeders, in a hierarchical mating system consisting of 30−35 males and 100 females (Gall and Neira, 2004; Gallardo et al., 2004). The fish used in this study correspond to a random sample drawn from a replicate that contained equal numbers of individuals of all the families of the breeding program. These fish had been freeze-branded as alevins at seven months of age (average weight=6 g) with a family identification mark, and stocked in rearing cages kept in brackish-water conditions in the Ensenada Baja Fjord (45° 30′S, 72° 50′W), where they remained for approximately 13 additional months until harvest.

### 2.2. Data and colour evaluation

Data on colour traits and harvest weights were recorded at 20 months of age for the 971 fish. The experimental population was divided into two pigment treatment groups, with families equally distributed within groups. The experimental groups were fed diets of different pigment content; the low pigment group received 35 ppm of the carotenoid astaxanthin, and the high pigment group received 70 ppm of the same pigment. Colour measurements were carried out using only visual methods in a light-controlled cabinet, with a cool blue fluorescent light source. Colour traits were measured in the standard Norwegian Quality Cut (NQC; Sigurgisladottir et al., 1997). Visual evaluation was made using two different standards: The Roche Colour Card for Salmonids (scores 1–8 and 11–18) and SalmoFan™ (scores 20–34). On both charts a high score indicates a deep red-orange colour.

### 2.3. Breeding value prediction

All visually scored colour traits were managed as continuous variables. We confirmed an underlying normal distribution using a Kolmogorov–Smirnov test

for normality (Zar, 1974). For prediction of breeding values we constructed an animal model (Eq. (1)). These values and genetic parameters were obtained using the derivative-free restricted maximum likelihood algorithm (DFREML; Graser et al., 1987) with the MTDFREML and DFREML programs (Boldman et al., 1995; Meyer, 1989). The general model in matrix notation was:

$$\boldsymbol{Y} = \mathbf{X}b + \mathbf{Z}a + \boldsymbol{e} \quad (1)$$

where $\boldsymbol{Y}$ is a vector of $n$ observations of colour; $\mathbf{X}$ and $\mathbf{Z}$ are design matrices for the fixed effects ($b$) and random additive genetic effects ($a$); and $\boldsymbol{e}$ is a vector of random residual effects. Fixed effects included in this model were: sex, due to sexual dimorphism which is reported in most carcass traits of salmonid fish (Crandell and Gall, 1993; Neira et al., 2004); pigment concentration in diet (70 and 35 ppm of astaxanthin); and harvest weight, included as a covariable since body size and colour traits are often correlated (Shearer, 1994). The expectation and variance matrices associated with Eq. (2) were assumed to be:

$$E\begin{bmatrix} b \\ a \\ \boldsymbol{e} \end{bmatrix} = \begin{bmatrix} \mathbf{X}b \\ 0 \\ 0 \end{bmatrix}; \mathrm{Var}[ai] = [\mathbf{A}gii] \text{ and } \mathrm{Var}[\boldsymbol{e}i] = [\mathbf{I}rii] \quad (2)$$

where $\mathbf{A}$ is the additive genetic relationships matrix between animals (i.e., the parenté coefficient of Malecot, 1948); $\mathbf{I}$ is the identity matrix; $gii = \sigma^2_{ai}$ and $rii = \sigma^2_{ei}$ are variances of additive genetic effects and residual effects for trait $i$, respectively. In the relationship matrix, pedigree information data for three generations of the experimental population were used; specifically these included grandparents, parents and offspring of salmon scored for colour traits.

### 2.4. DNA pool construction

Blood samples were collected from 971 individuals from the experimental population. All individuals were ranked based on their predicted breeding values (PBV) for flesh colour. As the ranking of PBV for all three colour scores were very similar, the average was used to make the final ranking. Thirty two individual samples were selected for DNA extraction, 16 fish with the highest and 16 fish with the lowest PBV for colour. DNA was isolated by phenol–chloroform extraction (Medrano et al., 1990) and quantified with a spectrophotometer (Hewlett Packard™; model 8452A). The high colour DNA pool (H) was prepared with a 5 ng/μl of genomic DNA from each of six salmon with the highest PBV for colour, while the low colour DNA pool (L) was prepared in similar way using six fish with the lowest PBV for colour.

### 2.5. RAPD analysis of potential markers

RAPD analysis was performed with 440 10-mer primers of arbitrary sequence obtained from the NAPS Unit of the University of British Columbia (primer sets 1 to 300) and Operon Technologies Inc. (primer sets: A, B, G, K, M, P and S ). PCR was performed in 15 μl volumes which contained 1.5 μl of 10 × PCR buffer, 100 μM of each dNTP, 0.4 μM primer, 1.8 mM $MgCl_2$, 0.5 U *Taq* DNA polymerase (Invitrogen™) and 20 ng of DNA from the colour pool. The thermal profile was 94 °C for 2 min, followed by three cycles at 94 °C (1 min), 35 °C (1 min), 72 °C (2 min), then 32 cycles at 94 °C (10 s), 35 °C (30 s) and 72 °C (1 min) with a final 5 min extension step at 72 °C. PCR reactions were performed using a MJ Research PTC-100 96-V thermal cycler. PCR products of both DNA pools were size separated in adjacent lanes by electrophoresis in a 2% agarose gel, stained with ethidium bromide and visualized under UV light. To determine the degree of association between polymorphic RAPD markers and colour breeding values, primers that amplified fragments in only one DNA pool were examined in all 32 individuals of both colour breeding value groups. The significances of associations were evaluated with Pearson's chi-square test (Choulakian and Mahdi, 2000).

### 2.6. Cloning and sequencing of RAPD fragments

DNA from RAPD markers associated with H or L pools was recovered from agarose gel with a QIAquick gel extraction kit (Qiagen™). The recovered DNA fragments were ligated in the plasmid vector pCR2.1TOPO using the TOPO-TA cloning kit (Invitrogen™) and were used to transform competent *E. coli* One Shot TOP-10 cells (Invitrogen™). For every marker, four clones were fully sequenced in an

automated DNA ABI Prism 310 Sequencer System (Applied Biosystem).

### 2.7. SCAR Primer design and colour-specific PCR in Coho salmon

For the cloned RAPD fragment, a pair of specific oligonucleotide primers was designed, converting the RAPD marker into a single locus PCR marker of a sequence-characterized amplified region (SCAR). Both specific primers contained the original 10 bases of the RAPD primer and the next 12–14 internal bases. SCAR amplification conditions in genomic DNA were 2 μl 10 × PCR Buffer, 100 μM dNTPs, 0.25 μM of each specific primer, 1.8 mM $MgCl_2$ and 0.5 U *Taq* DNA polymerase (Invitrogen™) in a final volume of 20 μl. The thermal profile was 94 °C for 2 min, followed by 29 cycles at 94 °C (1 min 30 s), 60 °C (1 min 30 s), 72 °C (2 min) with a 7 min extension step at 72 °C. This thermal profile was standardized according to the specific Tm of the primer pair. The PCR products were resolved by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide and visualized under UV light.

### 2.8. Association analysis of SCAR markers

The analysis of association of SCAR markers was performed using Pearson's $\chi^2$ statistic with one degree of freedom to test the null hypothesis that breeding value colour groups (H and L) were homogeneous with respect to the probability distribution of the presence of a SCAR marker (Choulakian and Mahdi, 2000).

Additionally, associations detected were tested in a second population of the same hatchery and with similar design of mating system and management procedures. From this population 849 salmons were evaluated for colour variables and ranked accordingly its PBV estimated in a similar way as it was performed on experimental population. In this case, 120 salmons (64 individuals with higher breeding values for colour and 56 for lower breeding values for colour) were selected for DNA extraction, and were tested for association analysis between SCAR markers and flesh colour, as has been previously described.

## 3. Results

### 3.1. RAPD screening

The initial screening of the 440 RAPD primers identified 29 potential polymorphic markers associated with high or low colour breeding values. These 29 primers were used for the individual screening of the 32 salmon samples from the experimental population. Only one primer (UBC 206, 5′-GAG GAC GTC C-3′) showed a consistent amplification pattern (fragment size approximately 550 bp) associated with high colour breeding value (Fig. 1). All 16

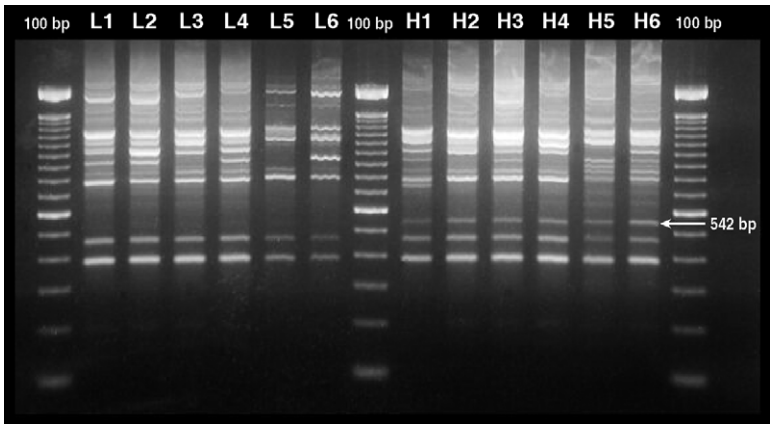


Fig. 1. RAPD amplification pattern obtained with UBC 206 primer in six individuals of high (H) and six individuals of low (L) colour breeding values. Arrows indicate the polymorphic marker associated with high colour breeding values.

high colour breeding value individuals in the experimental population amplified the RAPD marker and only 6 of 16 salmon of low colour breeding value showed the marker ($\chi^2=11.200$, $P<0.001$).

### 3.2. SCAR construction

The DNA fragment corresponding to RAPD marker UBC 206 was cloned and fully sequenced for its 542 bp (GenBank Accession No. AY661427). This SCAR did not show significant homology with any sequence in the NCBI database and it does not have any apparent difference in internal sequence between animals with high and low colour breeding values. Two specific long primers were synthesized based on the sequencing data: OKI206-1F(5′-GAG GAC GTC CTC CAA CCA ATC A-3′) and OKI206-518R (5′-GAG GAC GTC CTC ACC TTA CAA TCC-3′). These primers were used to amplify the *Oki*206 SCAR marker in all 32 individuals from the experimental population (Fig. 2); the amplification showed a dominant presence/absence pattern. The amplification profile was optimized at the annealing temperature of 68 °C. *Oki*206 was amplified in 14 of the 16 salmon with high colour breeding values and only 2 of 16 individuals with low colour breeding values ($\chi^2=21.208$, $P<0.0001$). This association pattern was corroborated in the 120 salmon samples from the second population, where the frequency of this marker was of 76.6% in individuals with higher breeding value and 37.5% in individuals with lower breeding value ($\chi^2=18.761$, $P<0.0005$).

## 4. Discussion

This investigation combined the use of classical breeding value prediction with selective DNA pooling using anonymous RAPD molecular markers, screening to detect markers associated with a quantitative trait of low heritability (estimates of $h^2$ for flesh colour obtained in this study varied from 0.20 to 0.23, and will be reported elsewhere). In mathematical models proposed for the identification of quantitative trait loci, it is assumed that the phenotypic value is a good predictor of the genotypic value, without taking into account that transmission values are the main source of variation in complex traits (for instance, Lander and Botstein, 1989; Darvasi and Soller, 1992). The approach used here to build the *Oki*206 marker makes use of the best genetic prediction linked to the genes that are influencing the expression of colour. We infer that *Oki*206 co-segregates with one or more loci with an important contribution to breeding values for flesh colour.

The amplification of *Oki*206 was not always positive in individuals from the H group; a small proportion of high colour breeding value salmon did not amplify this SCAR. This result is not unexpected, due to the nature of the polygenic architecture of quantitative traits. In this way, it is possible that some individuals may have a high flesh colour score due to alleles at other loci influencing this trait and it does not present co-segregation with the marker, as has been shown in some individuals. However, our data suggest the presence of a QTL co-segregating with

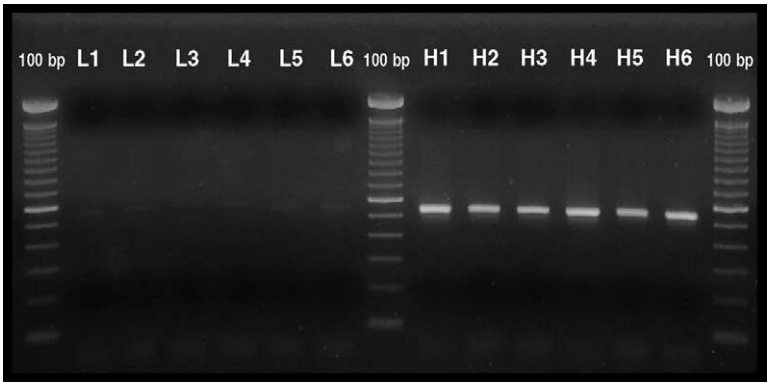


Fig. 2. Amplification of SCAR *Oki*206 in six individuals of high (H) and six individuals of low (L) colour breeding values.

*Oki*206, although the evidence of this association needs to be confirmed with a linkage study or with a co-selection study in other hatchery populations (Abiola et al., 2003).

The co-selection of this marker may be evaluated experimentally in other breeding programs. Due to its presence/absence amplification pattern, *Oki*206 may be used as a fixed effect in an animal model (Meuwissen and Goddard, 1997) that combines the use of phenotypic data to carry out a main selection objective, for instance growth rate, and molecular information for a secondary selection objective, such as flesh colour.

Several microsatellite markers have been associated with quantitative trait loci in salmonids, such as upper thermal tolerance (Jackson et al., 1998; Danzmann et al., 1999), spawning time (Sakamoto et al., 1999; Fishback et al., 2000) and resistance to IPN virus (Ozaki et al., 2001), however to date there is no published information of a breeding program that includes molecular information to select fish. The application of MAS in a breeding program has an additional cost of genotyping at least 500 individuals every generation. Under this scenario, the use of a dominant marker, like *Oki*206, has the advantage of the possible development of a less expensive, quick, plus/minus calorimetric assay to detect the marker, thus avoiding the need for electrophoresis (Paran and Michelmore, 1993), as has been developed to sex bovine embryos (Bredbacka et al., 1995, 1996).

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