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Short Communication

An evaluation of a diagnostic test to identify the sex of farmed rainbow trout, using sex-specific molecular markers

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ABSTRACT. In trout farming males exhibits lower growth rate and precocious sexual maturation in contrast to females. Since these traits are detrimental for intensive production, all females populations are most appreciated by fish farmers. Molecular markers sex-specific have been developed for rainbow trout that can be useful to diagnose the phenotypic sex of individuals. We evaluated the use of two SCAR markers (OmyP9 & Omy163), which show polymorphisms between males and females in rainbow trout as a diagnostic test for sexing farmed trout. Adult trout (n = 131) were genotyped to assess the association of the SCAR markers with phenotypic sex. To evaluate the correct performance of the SCAR marker in sex diagnosis, each marker and both were analyzed to estimate its specificity (the proportion of males that are correctly identified), sensitivity (the proportion of females that are correctly identified) and predictive value (the probability of the correct positive or negative female identification). Significant associations with phenotypic sex of both SCAR markers with sex were found. The sensitivity and predictive (positive and negative) indexes show higher values when both SCAR markers were considered (95.7, 77.4 and 94.4%, respectively). For joined SCAR markers a likelihood ratio (*LR+*) of 3.43 was obtained indicating its utility to establish a diagnostic test for sexing trout by use of marker-based analysis.

Keywords: *Oncorhynchus mykiss*, OmyP9, Omy163, sex marker, diagnostic test.

Evaluación de un test diagnóstico para identificar el sexo en truchas arcoíris por medio de marcadores moleculares específicos

RESUMEN. En el cultivo de truchas los individuos del sexo masculino presentan menor tasa de crecimiento y maduración sexual precoz, características que no presentan las hembras. Debido a estos rasgos indeseables para el cultivo intensivo, el uso de poblaciones todo hembra es una práctica habitual en el cultivo de truchas. Para trucha arcoíris se han desarrollado marcadores moleculares sexo específico que pueden utilizarse en el diagnóstico temprano del sexo, en condiciones de cultivo. Se evaluó la aplicación de dos marcadores SCAR (OmyP9 y Omy163), que muestran polimorfismos entre los sexos en esta especie. Con ambos marcadores se genotipificaron 131 truchas para evaluar su asociación con el sexo fenotípico. Para determinar la utilidad en diagnóstico del sexo de cada marcador SCAR por separado, y en conjunto, se determinó la especificidad, sensibilidad y valores predictivos en la detección de un individuo de sexo femenino. Se comprobó la asociación significativa descrita para ambos SCAR con el sexo fenotípico. La mayor sensibilidad y valores predictivos positivo y negativo se obtuvo usando ambos marcadores simultáneamente (95,7; 77,4 y 94,4%; respectivamente). El valor de razón de verosimilitud (*LR+* =3,43) mostró que la mejor prueba de diagnóstico para detectar hembras es usar conjuntamente OmyP9 y Omy163.

Palabras clave: *Oncorhynchus mykiss*, OmyP9, Omy163, marcador de sexo, test diagnóstico.

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Fish have different reproductive strategies, where the chances of reproduction are based on the sex and size of individuals, often showing sexual dimorphism for growth (Parker, 1992; Brantley *et al.*, 1993). Individuals of one sex have better production characteristics than those of the other sex, such as faster growth or delayed sexual maturation. Increased growth can occur in either sex, but in most cultured fish species, females show higher growth rates than males (Piferrer *et al.*, 2007).

In rearing conditions, sex-related growth generates excessive size dispersion during fattening. Hence, size

classifications or unfolding must be performed in order to avoid cannibalism or size hierarchies affecting social relations (Dou *et al.*, 2004). This represents more work in animal husbandry, increased need for labor, and an increased number of production units to accommodate different growth groups (Piferrer *et al.*, 2007).

In addition to the problem of differential salmon growth between sexes, variability on the sexual maturation onset also exists (Zanuy *et al.*, 2001). Early maturation in salmonids occurs differentially in males and females and creates negative outcomes for production (Felip *et al.*, 2006). Precocious fish present reduced growth, since much of the food energy is spent on the maturation of gonads and gamete formation (Thorpe, 1994). Also, there is an increased susceptibility to diseases (Schreck *et al.*, 2001) and a significant change in organoleptic properties of the edible parts, due to the alteration of fat stores and their lipid profiles (Johnston *et al.*, 2000).

Sex determination in salmonids is regulated by factors contained in specific chromosomes. In rainbow trout and other salmonids, the sex determination system corresponds to the XY system, being the sex chromosomes morphologically undifferentiated (no heteromorphism between X and Y chromosomes) or small differences (Thorgaard, 1977; Hartley, 1987; Oliveira *et al.*, 1995; Colihueque *et al*., 2001; Iturra *et al.*, 2001b; Ocalewicz *et al*., 2007). Molecular methods have allowed the development and characterization of sex-specific markers, which are useful for sex diagnosis at any stage of development. To date, a number of molecular markers have been developed for sex identification in Pacific salmon (Devlin *et al.*, 1991; Noakes & Phillips, 2003; Brunelli & Thorgaard, 2004), brown trout and Atlantic salmon (Li *et al.*, 2011), and rainbow trout (Iturra *et al.*, 2001a; Felip *et al.*, 2005). However, these markers have not been extensively used for sexing salmons in practical cultures. The aim of this study was to evaluate two sex associated SCAR markers (Sequence characterized amplified regions) to assess the phenotypic sex in farmed rainbow trout. A SCAR marker is a PCR-based marker representing a single genetically defined locus (Paran & Michelmore, 1993).

We studied a rainbow trout family composed of 131 individuals obtained from a cross between a F_1 female (Wytheville strain x Steelhead strain) and a Steelhead strain male. Genomic DNA was extracted using the phenol chloroform method (Taggart *et al.*, 1992), from fin clip samples obtained at the alevin stage for all individuals in 2008. All alevins were tagged with an electronic tag (passive integrator transponder or PIT-tag) allowing correlating the phenotypic sex (determinated at the first spawning time, between April and June 2010) with the genotypic data.

We used two SCAR markers (OmyP9 and Omy163) located on the rainbow trout sex cromosomes, which shows polymorphisms between sexes. SCAR OmyP9 (Iturra *et al.*, 2001a) shows size polymorphisms and a different RFLP pattern between males and females, while SCAR Omy163 (Felip *et al.*, 2005) is linked to the Y sex chromosome, where allele segregation allows us to identify the sex of the offspring. For OmyP9, the B genotype corresponds to female and the AB genotype is male (Fig. 1a), while for SCAR Omy163, the C genotype corresponds to females, while D and E genotypes represent male (Fig. 1b).

OmyP9 was amplified with the PCR reaction mix modified from Iturra *et al.* (2001a): 1x PCR Buffer, 2 mM $MgCl_2$, 0.1 mM dNTPs, 0.3 µM of each primer, 0.5 U of recombinant Taq DNA Polymerase (Fermentas®) and 40 ng of DNA, in a final volume of 15 µL. The amplification profile consisted of an initial denaturation step, at 94°C for 2 min, followed by 30 cycles at 94°C (45 s), 55°C (45 s) and 72°C (45 s), and a final elongation step at 72°C for 10 min. Then, 10 µL of this reaction were digested with 2 U of *RsaI* (BioLabs ®) in a final volume of 14 µL at 37°C for 2 h.

The PCR reaction mix for Omy163 consisted of: 1x PCR Buffer, 4 mM $MgCl_2$, 0.2 mM dNTP, 0.2 mM of each primer, 0.9 U of recombinant Taq DNA Polymerase (Fermentas ®) and 40 ng of DNA in a final volume of 15 µL. The amplification profile in this case had an initial denaturation step at 94°C for 3 min, followed by 30 cycles at 94°C (50 s), 58°C (50 s) and 72°C (50 s) and final elongation step at 72°C for 2 min. Digestion products for OmyP9 and the amplification of Omy163 were visualized on agarose gels 1.5% p/v, stained with ethidium bromide.

The level of association between the gender on the individual obtained with both molecular markers and the gender obtained at the phenotypic level was assessed with a Fisher's exact probability test (Zaykin & Pudovkin, 1993). Furthermore, to determine its usefulness in sex diagnosis, specificity and sensitivity to detect a female trout (positive assignation) was determined for each marker (Altman & Bland, 1994a). In this case Sensitivity is the proportion of true (phenotypic) females that are correctly identified by the SCAR markers and Specificity is the proportion of true males that are correctly identified by these markers.

$$Specificity = \frac{P(MG \cap MP)}{P(MP)}, \quad Sensitivity = \frac{P(FG \cap FP)}{P(FP)}$$

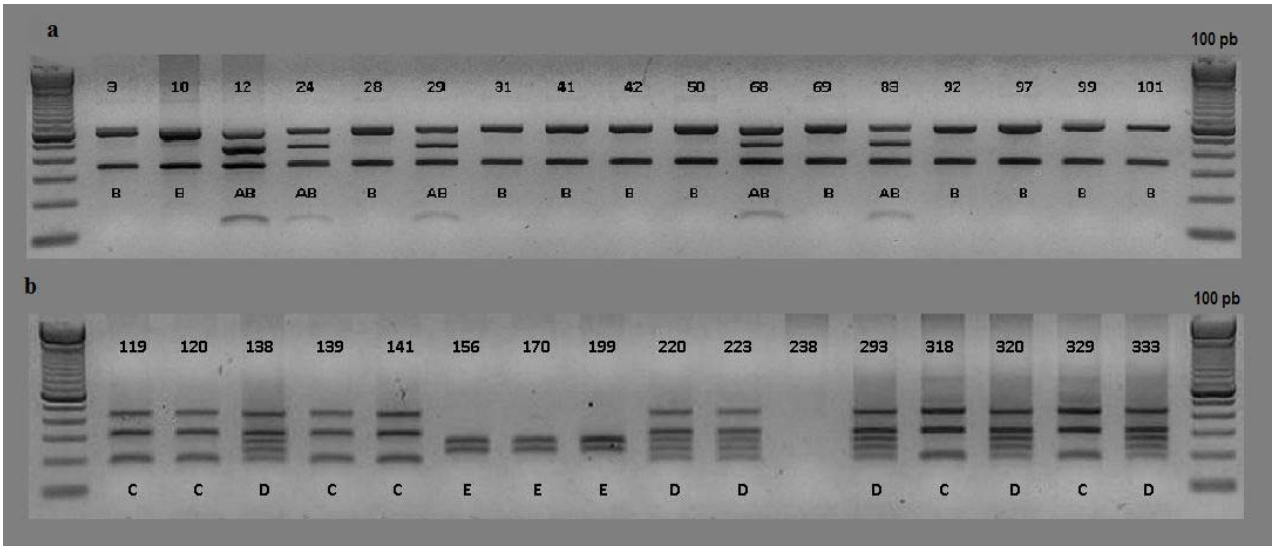

Figure 1. a) SCAR OmyP9 amplification in 17 rainbow trout individuals, b) SCAR Omy163 amplification in 15 rainbow trout individuals.

Figura 1. a) Amplificación del SCAR OmyP9 en 17 individuos de trucha arcoíris, b) amplificación del Omy163 SCAR en 15 individuos de trucha arcoíris.

Where; *MP* is male phenotypic sex, *FP* is female phenotypic sex, *MG* is male genotypic sex and *FG* is female genotypic sex, obtained with SCAR markers.

To evaluate the probability that these SCAR markers will give the correct gender identification, positive predictive value (*PPV*), *i.e.*, the females rate with positive results correctly sexed, and negative predictive value (*NPV*), *i.e.*, the females rate with negative results correctly sexed, were estimated using the frequency of female (0.5), in a normal population, as the Prevalence (Altman & Bland, 1994b).

$$PPV = \frac{Sensitivity \cdot Prevalence}{Sensitivity \cdot Prevalence + (1 - Specificity) \cdot (1 - Prevalence)}$$

$$NPV = \frac{Specificity \cdot (1 - Prevalence)}{(1 - Sensitivity) \cdot Prevalence + Specificity \cdot (1 - Prevalence)}$$

The analysis of Specificity, Sensitivity, *PPV* and *NPV* was also performed on selected females and males that only exhibited both markers positive for the respective sex. Finally, the value of the likelihood ratio (*LR+*) was assessed to evaluate the usefulness of the molecular SCAR markers for sex diagnosis (larger values indicate greater utility of the test):

$$LR+ = \frac{Sensitivity}{(1 - Specificity)}$$

The association analysis showed a significant level of association ($P < 0.0001$) of OmyP9 and Omy163 SCAR markers with phenotypic sex (Table 1), which agrees with data reported previously (Iturra *et al.* 2001a; Felip *et al.* 2005). The sensitivity, specificity and predictive values of both markers individually and jointly are shown in Table 2. OmyP9 showed higher Specificity (74.5%). On the other hand, when both markers were used together, Sensitivity increased to 95.7%, along with *PPV* and *NPV*, with values of 77.4% and 94.4% respectively. Likelihood ratio ranges from 2.66 for Omy163 to 3.43 for both markers used altogether, the higher the value of *LR+* indicate greater utility of the test for diagnosis. Therefore, the joint application of both sex markers (Omy163 and OmyP9), allows us to differentiate reliably the phenotypic sex of individuals before this trait was expressed. This result indicates that, in a hatchery population, males can be effectively identified to be discarded from culture prior to their sexual maturity using Omy163 and OmyP9 SCAR markers. An interesting application of this diagnostic test is to be used in neomales offspring sexing, as a verification of genotypic female sex. A neomale is an individual genetically female (XX), which has been masculinized using androgens previous to gonadal differentiation, with 17α-methyl testosterone for example. These neomales are used in hatcheries to be mated with a normal female in order to produce all female fertilized eggs. A usual practice is to produce neomales from a normal population, with a mix of male and female individuals. In such conditions a molecular sexing test is useful to discard normal males (XY) from neomales (XX), before fertilization.

Table 1. Association analysis between real phenotypic sex and sex determined by OmyP9 and Omy163 markers in rainbow trout.

Tabla 1. Análisis de asociación entre el fenotipo sexual real y el sexo determinado por los marcadores OmyP9 y Omy163 en trucha arcoíris.

Phenotypic Sex	OmyP9 AB(♂)	B (♀)	*P*	Omy163 D and E(♂)	C (♀)	*P*
Male	35	12	0.00001	31	16	0.0001
Female	12	72		8	76	

Table 2. Sensitivity, specificity and predictive values from SCAR marker-based sexing tests for rainbow trout. PPV: positive predictive value, NPV: Negative predictive value, LR: likelihood ratio.

Tabla 2. Sensibilidad, especificidad y valores predictivos obtenidos desde test de sexados basados en marcadores SCAR para trucha arcoíris. PPV: valor predictivo positivo, NPV: valor predictivo Negativo, LR: razón de verosimilitud.

Marker	Specificity	Sensitivity	*PPV*	*NPV*	*LR+*
OmyP9	0.7446	0.8571	0.7704	0.8390	3.36
Omy163	0.6596	0.9048	0.7266	0.8739	2.66
OmyP9+Omy163	0.7209	0.9571	0.7742	0.9438	3.43

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