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Short communication

Development and validation of a molecular tool for assessing triploidy in turbot (*Scophthalmus maximus*)

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ABSTRACT

Production of triploid individuals is a relevant goal for the aquaculture industry due to the benefits associated with their sterility and growth. Thus, methods for assessing triploidy have been developed based on genome, chromosome or gene triploid-associated properties. In this study, we developed a new cheap, technically simple and accurate method to validate triploidy in turbot (*Scophthalmus maximus*) based on microsatellite markers. Five crosses were performed to produce diploid and triploid progenies that were used to validate this molecular tool. Flow cytometry, one of the most widely used and accurate techniques for ploidy determination, was used as reference to contrast results. A set of four highly polymorphic and largely distant to centromere microsatellites was selected for this purpose. Ploidy was easily evaluated according to the maximum number of alleles at the microsatellite loci tested, diploids showing two and triploids three. These microsatellites were combined in a single multiplex and were able to identify triploids with 100% accuracy in all analyzed crosses.

1. Introduction

The turbot (*Scophthalmus maximus*) is a commercially valuable flatfish species, being one of the most promising marine species of European aquaculture. Its farming production has experienced an increasing demand in the last decade (7702 tonnes in 2009; 83.3% European production from Spain; Apromar, 2010), and an important production increase is predicted in the next years (more than 18,000 tonnes in 2014; FEAP, 2010). Commercial size, around 1800–2000 g, is achieved at approximately 2 years. At this age the onset of sexual maturation occurs, and as in other fish, it involves a reduction in somatic growth and higher mortalities (Cal et al., 2006). Thus, production of sterile populations by chromosome set manipulation is a research line of interest for improving turbot culture, allowing larger sizes of high commercial value (Piferrer et al., 2000).

Triploidy has been induced in an important number of marine species, although its final application in fish farms has been limited (Aloise et al., 2011; Felip et al., 2001; Piferrer et al., 2009). Triploid individuals contain three chromosome sets, and they are generally sterile, thus avoiding the undesirable effects of maturation, and also the genetic impact of escapees of farmed individuals on wild populations (Aloise et al., 2011; Piferrer et al., 2009). Triploidization is the consequence of suppression of the second polar body extrusion and is achieved by temperature or pressure shocks of fertilized eggs. Temperature shock treatments are inexpensive to apply and can be successfully adapted for mass production by fish farms (Piferrer et al., 2003). Triploid turbot have been obtained by cold shock (Piferrer et al., 2000), and in addition to their sterility and higher growth after sexual maturation (Cal et al., 2006), triploids are mostly females, representing an additional advantage because females largely outgrow males in this species.

Production of triploids necessarily requires a method for validation. Triploid fish have been identified by indirect methods such as measurement of nuclear and cellular size of erythrocytes (Purdom, 1993), counting of nucleoli (Howel and Black, 1980), electrophoresis of proteins and examination of morphology (Liu et al., 1978). Direct methods include chromosome counting by karyotype analysis and DNA content determination by flow cytometry (Thorgaard, 1983). Each technique has advantages and drawbacks, and the choice of a method for determining ploidy depends on its accuracy, and the objectives and cost considerations of the study. Karyotyping is the only irrefutable technique to determine ploidy. However, it is also the most time-consuming and frustrating one, which reduces its applicability in mass screening of fishes (Harrel and Van Heukelem, 1998). Erythrocyte nuclear and cellular size could be considered an alternative approach to chromosome scoring. However, range overlap between ploidy levels often occurs for each variable, and the

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technique is not possible at all ages (Wolters et al., 1982). Silver staining of nucleolar organizer regions (NORs) is a straightforward technique for verifying ploidy when no polymorphism of number exists, but NORs are sometimes difficult to detect and there are conflicting results in older fish (Harrel and Van Heukelem, 1998).

Allen (1983) compared various techniques and proposed that flow cytometry was the most effective one. Flow cytometry is a powerful technique for estimating nuclear DNA content because it permits sensitive measurements of fluorescence intensity of a large number of stained nuclei within a short time (Sari et al., 1999). This technique is one of the most widely used and it has been applied for ploidy determination in several fish species (Allen and Stanley, 1983; Chao et al., 1993; Ewing et al., 1991; Lamatsch et al., 2000; Thorgaard et al., 1982). However, it can be only applied to fish whose size allows blood cell extractions and with some risk because it is rather invasive being able to damage fish. In addition, flow cytometry requires highly specialized equipment and a certain technical competence to perform the sample preparation.

Triploidy validation in turbot has been performed by nucleoli counting (Piferrer et al., 2000), erythrocyte size measurement (Piferrer et al., 2003) and flow cytometry (Vázquez et al., 2002). The first two methods showed a certain error because of overlapping of diploid vs triploid distributions and the three techniques are invasive. Additionally, the last two methods require samples of a certain age with an appropriate amount of blood cells for analysis. Thus, a cheap technique that is non-invasive and can be performed at any age starting from small tissue samples would be interesting for testing triploidy in turbot.

The development in the last years of genomic resources has provided a large amount of highly polymorphic genetic markers, such as microsatellites, useful for individual identification and pedigree tracing in fish (Castro et al., 2006; Chistiakov et al., 2006; Pino-Querido et al., 2010). Several parameters like polymorphism, frequency of null alleles and accurate genotyping are essential to assess the potential and accuracy of microsatellites for parentage assignment (Castro et al., 2004). To date, close to five hundred microsatellites have been reported in turbot (Bouza et al., 2002; Coughlan et al., 1996; Estoup et al., 1998; Iyengar et al., 2000; Pardo et al., 2005, 2006, 2007; Ruan et al., 2010). The development of a genetic map in this species (Bouza et al., 2007) and the localization of centromeres (Martínez et al., 2008) allow knowing the position of markers and estimating their recombination frequency to centromeres. If the distance between a specific marker and the centromere is high, crossover between homologous chromosomes will take place at prophase I of most meioses. If both parents do not share any alleles at a specific marker, triploids will show three different alleles, being easily identified. Thus, highly polymorphic and centromere-distant microsatellites offer the opportunity to develop a straightforward molecular method to validate triploidy.

The aim of this work was to develop a cost-effective, non-invasive, versatile and accurate molecular tool starting from a set of highly polymorphic centromere-distant microsatellite loci for detection of triploids in turbot. The performance of this tool was compared with flow cytometry, one of the most accurate techniques for ploidy determination.

2. Materials and methods

2.1. Families and triploidy induction

Fish used in this experiment were reared at the facilities of the Spanish Institute of Oceanography in Vigo (NW Spain) in 2009 and 2010. Five experimental crosses (I to V) were performed using eggs and sperm from a couple, excluding cross I, where three males and one female were used (Table S1). In each cross, fertilized eggs were divided into two groups. In one group triploidy was induced after fertilization by cold shock according to Piferrer et al. (2000). The other group was not treated and was used as diploid control. Global performance of the triploidy induction was verified at each cross by nucleolar organizing region (NOR) analysis in 30 two-day-old larvae (Piferrer et al., 2000). More than 95% triploids were obtained in each experimental cross. Moreover, a blind sample of 28 induced-to-triploid individuals for a cross performed using one female and two males with unknown genotypes was analyzed in order to validate our molecular tool (see below). For each cross, turbot larvae were reared according to the standard protocol for this species (Olmedo, 1995). Treated (3n) and control (2n) larvae were reared separately in two 1000-l tanks at 18 °C and fed with rotifers and Artemia until the end of metamorphosis, between 30 and 40 days of life. Thereafter, diploids and triploids were placed in two separate 3800-l tanks provided with flow-through water, and fish were reared under natural conditions of photoperiod and temperature. Fish were fed by automatic feeders with dry pellets of increasing size (Skretting, Burgos, Spain) 7 days a week until the end of the experiment. Ploidy was examined in 8–15 month-old fishes by flow cytometry for an accurate individual validation. The following expected triploid and diploid individuals, respectively, were taken at each cross: 25 and 20 at cross I; 35 and 21 at cross II; 25 and 39 at cross III; 30 and 30 at cross IV; 30 and 30 at cross V.

2.2. Ploidy determination by flow cytometry (FC)

Fish were anesthetized with MS-222. Blood was collected with heparinized syringes from the caudal vein or in some cases from gills. Approximately 5 μl of blood cells was added to 1.5 ml of ice cold 70% alcohol, mixed and stored at −20 °C. The protocol by Darzynkiewicz et al. (1997) was applied for ploidy estimation. Briefly, the collected cells were washed twice with cold phosphate buffer saline (PBS) and then resuspended in 1 ml of PBS. Finally, 50 μl of cells was stained by adding 450 μl of 0.1% Triton X-100 in PBS with 50 μg/ml propidium iodide. The cellular fluorescence was measured using a Coulter FACS flow cytometer (FC500MPL) and the WinMDI 2.9 software (Trotter, 2000). To asses triploidy we determined the mean fluorescence intensity (GMean) and calculated the ratio sample-GMean to 2n-GMean. We considered triploid if the ratio was ~1.5.

2.3. DNA extraction, PCR amplification and microsatellite genotyping

DNA was extracted using the Chelex® Resin procedure (Walsh et al., 1991) from caudal fin samples obtained from used breeders and progenies. A set of seven highly polymorphic (Pardo et al., 2006, 2007), unlinked (Bouza et al., 2007) and far from centromeres (Martínez et al., 2008) microsatellite loci (Sma-USC12, Sma-USC24, Sma-USC27, Sma-USC29, Sma-USC31, Sma-USC113 and Sma-USC227, see Table 1) were amplified following the PCR conditions

Table 1
Characteristics for microsatellite loci used in this study indicating the number of alleles (A), gene diversity (He), heterozygote frequency (y) and frequency of null alleles (F_{null}).

Loci	A	He	y	F_{null}
Sma-USC12	17[a]	0.932[a]	0.958[b]	−0.001[a]
Sma-USC24	16[a]	0.913[a]	0.848[b]	−0.036[a]
Sma-USC27	14[a]	0.834[a]	0.771[b]	0.055[a]
Sma-USC29	10[a]	0.809[a]	0.854[b]	0.016[a]
Sma-USC31	9[a]	0.772[a]	0.646[b]	0.060[a]
Sma-USC113	9[c]	0.751[c]	0.933[b]	0.035[c]
Sma-USC227	17[c]	0.931[c]	0.913[b]	0.105[c]

[a] From Pardo et al. (2006).
[b] From Martínez et al. (2008).
[c] This study using the same 24 wild Cantabric individuals analyzed in Pardo et al. (2006).