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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).

reported by Pardo et al. (2006, 2007). Their fluorescence labeling is shown in Table S1.

After checking the performance of all loci used (presence of null alleles and genotyping errors), the best four microsatellite loci were amplified together in a single multiplex PCR (Sma-USC12, Sma-USC24, Sma-USC29 and Sma-USC113). No formation of hairpin and dimers among used primers was checked using AutoDimer Version 1.0 (Vallone and Butler, 2004). Loci were amplified in 10 μl volumes which consisted of 0.8 μl template DNA (~30 ng) in 1X QIAGEN Multiplex PCR Master Mix (containing HotStar Taq DNA Polymerase, Multiplex PCR Buffer kit, $MgCl_2$, dNTP mix, 0.2 μM of both forward and reverse PCR primer for Sma-USC12, Sma-USC24 and Sma-USC113 and 0.3 μM of both forward and reverse PCR primer for Sma-USC29). Thermal cycling was conducted on a Verity™ 96-Well Thermal Cycler (Applied Biosystems) as follows: initial denaturation at 95 °C for 15 min, 35 cycles of denaturation at 94 °C for 30 s, 50 °C (annealing multiplex temperature) for 90 s, and extension at 72 °C for 90 s. There was a final extension step at 60 °C for 30 min. PCR products were resolved by using an ABI PRISM® 3730 automatic sequencer (Applied Biosystems). Allele scoring was performed with GeneMapper 4.0 software (Applied Biosystems). Individuals with three alleles in at least one of the four microsatellite loci tested were considered triploids.

Cervus 3.0.3 software (Kalinowski et al., 2007) was used to estimate the number of alleles (A), gene diversity (He) and the frequency of null alleles at Sma-USC113 and Sma-USC227 loci (Table 1). Parent pair analysis was conducted to assign parents to offspring at cross I (one female and three males involved) using the exclusion method implemented in Cervus software (Kalinowski et al., 2007). The genotypes of candidate parents were compared against the offspring's genotype, and candidates were excluded if a mismatch occurred at one or more loci. For triploid individuals, the two maternal alleles were coded as a single allele to perform the analyses.

3. Results and discussion

The three expected families were represented at cross I, but an important bias on male contributions was detected using the seven microsatellite loci. All individuals were undoubtedly assigned to one mating pair: 84.5% (38 out of 45 individuals) of the progeny were assigned to the Male 44 × Female 23 mating pair (14 individuals representing the 70% of diploid progeny and 24 individuals representing the 96% of triploid progeny); 11% (5 individuals) to Male 32 × Female 23 pair (4 individuals representing the 20% of diploid progeny, and 1 individual representing the 4% of triploid progeny); and only 4% (2 individuals) to Male 4 × Female 23 pair (2 individuals representing the 10% of diploid, no triploids assigned). These results confirm the utility of the loci tested for parentage analysis. Six triploid individuals were detected in the control tanks of diploid individuals (see Table S1) with a mishandling error rate of 4.3% (6 triploids detected in control diploid tanks/140 sampled). The presence of these triploids is likely due to an exceptional incorrect larvae handling by the personal in the plant, since no spontaneous triploids have been recorded in usual culture conditions.

The methods applied so far to validate triploidy in turbot showed a certain error or required blood cells, and thus, were only applicable on adult individuals (Cal, 2005; Piferrer et al., 2000). Flow cytometry is the most accurate technique for assessing triploidy in turbot. Unfortunately, the flow cytometry equipment is not routinely available due its high cost associated with purchase and maintenance, and the need for trained technicians to operate the equipment. In addition, this method shows some technical limitations. In this study we developed a non-invasive, fast, accurate, and cost-effective method to validate triploidy in turbot at any age stage based on microsatellite markers. Markers were chosen according to the following criteria: i) high polymorphism (more than 9 alleles and He > 0.750); ii) low null allele frequency (<0.100); iii) distant from centromeres (heterozygote frequency in gynogenetic progeny (y) > 0.650); and iv) no genotyping errors according to available information (Martínez et al., 2008; Pardo et al., 2006, 2007). With these criteria and using an appropriate set of microsatellites, triploids should be easily identified by the presence of three-allele genotypes. This circumstance would be enhanced in turbot because full interference is the best fitted mapping function (Martínez et al., 2008), and thus the formation of two or multiple crossovers which could produce homozygotes in centromere-distant markers is unlikely. This would be nearly impossible at several markers when they are located in different linkage groups, as occurs with our molecular tool.

Despite that the estimated frequency of null alleles on the analyzed loci was low (F_{null} < 0.100; Pardo et al., 2006, this study), we could detect their presence at Sma-USC27, Sma-USC31 and Sma-USC227 markers after family analyses in the performed crosses. These results confirm their estimated frequency from a wild population, since these three markers showed the highest null allele frequency (Table 1), and suggest that a more restrictive null allele frequency threshold should be used (i.e. F_{null} < 0.050). Null alleles are non-amplified alleles that, when segregating with another allele, result in an apparent homozygote, thus strongly hampering detection of triploids with our method. For this reason, these three loci were excluded for the development of our multiplex tool.

If shock treatment is not 100% effective, cohorts of putative triploids will contain diploids that failed to respond to the shock treatment. On the other hand, two-allele genotypes that could interfere with the analysis may appear in triploids by three different causes: i) the mother is homozygous for that locus; ii) both parents share one or two alleles in that locus; and iii) there was no recombination between the analyzed locus and the centromere in the mother. The potential failure of detecting triploidy due to points i) and ii) is known at each locus by simple inspection of parent genotypes and depends on microsatellite polymorphism, inbreeding and relatedness between parents. On the other hand, the lack of recombination is a probability always existing at any locus which depends on its distance to the centromere. Thus the probability of detecting triploids by three-allele genotypes mainly relies on checking a sufficient number of highly polymorphic and distant to centromere microsatellite loci. Additionally, the appropriate management of the broodstock would aid to avoid an excess of homozygotes due to inbreeding, but at any case the effectiveness of our tool relies on the use of several markers and only in highly inbred lines ploidy discrimination would be precluded. The chromatogram patterns showing three different alleles are easily identified, because individuals show three different peaks (each of them corresponding with one specific allele). Individuals with two alleles in one locus (heterozygous) show two different peaks and homozygous individuals show only one peak. The three-peaks/alleles patterns, used for the detection of triploid individuals, were observed in the seven tested microsatellite loci in cold-shocked fish (Fig. 1A, Fig. S1). Some individuals showed a different ploidy than that expected according to the treatment applied, and this circumstance was detected both with flow cytometry and the molecular tool. Only in cross IV all individuals showed the expected ploidy (Table 2). Correspondence between both techniques (cytometry and molecular tool) was 100%, except for cross III, where three individuals assigned as triploid by flow cytometry only showed two alleles (peaks) with our molecular tool (Table 2). The percentage of triploid detection using the molecular tool was 97.9% when compared flow cytometry data. Thus, 143 individuals among 146 assigned as triploids by flow cytometry showed three different alleles at least in one of the microsatellite loci studied. This value was higher than those described in Chinook salmon (*Oncorhynchus tshawytscha*) using seven microsatellite loci (72.5%; Garner et al., 2008).

For most microsatellite markers, shorter alleles are amplified with higher intensity than longer ones due to their shortest PCR reaction.

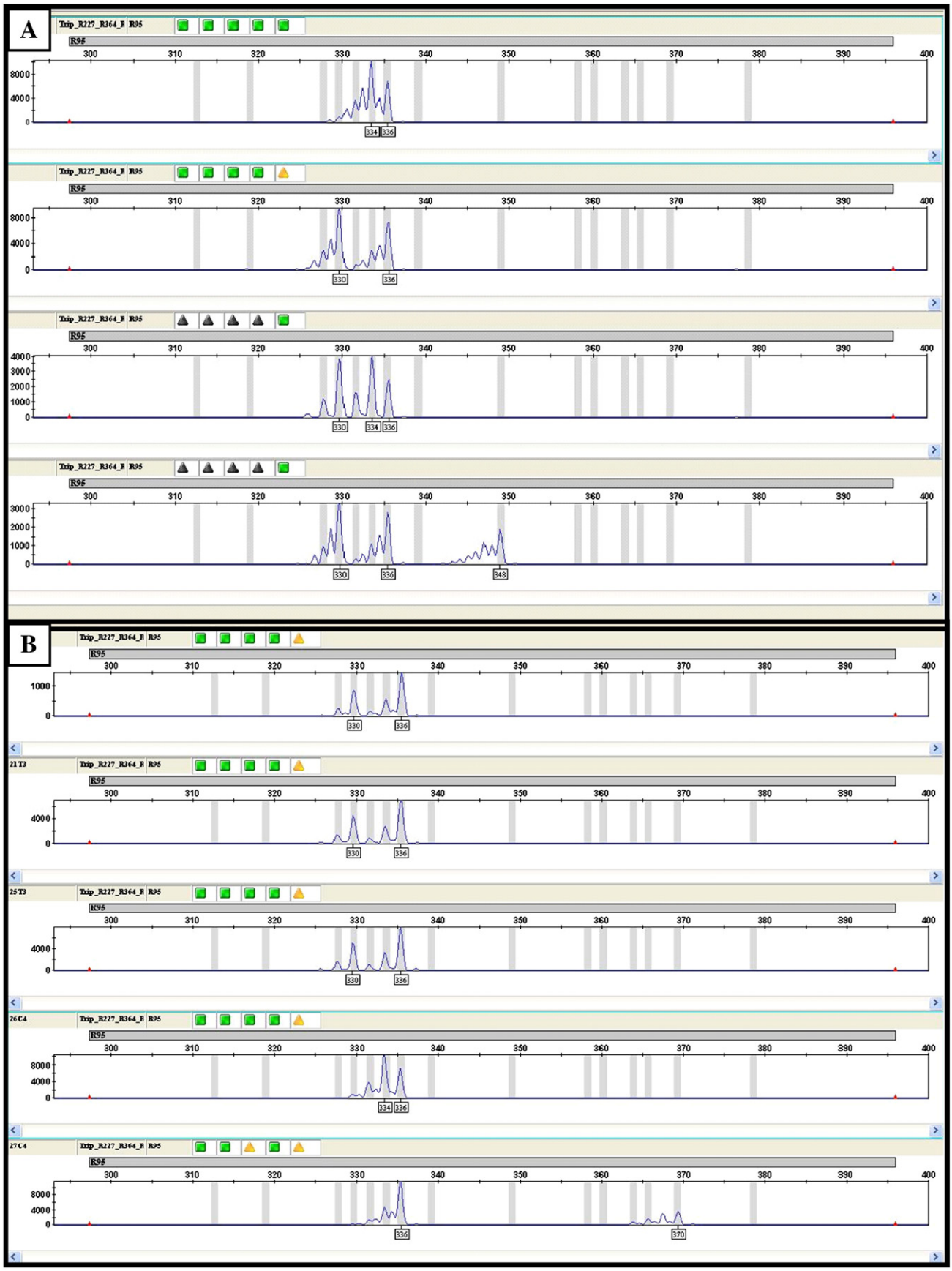

Fig. 1. Detection of triploid individuals for Sma-USC12 marker. A) The two upper and the two lower chromatograms correspond with diploid and triploid individuals, respectively. B) Detection of triploids showing only two peaks in family III. The three upper chromatograms correspond with the three unassigned triploids, and the two lower chromatograms correspond with diploids.

Table 2
Results for individuals induced to triploidy. "N" corresponds with the number of individuals analyzed, "Expected" corresponds with the expected ploidy of the individuals used (see text), "Cytometry" corresponds with the observed results for this technique, "Molecular tool" corresponds with the ploidy observed using the multiplex microsatellite tool developed in this work, and "% Correct assignment" is the percentage of correspondence between both techniques. Numbers between parentheses indicate individuals expected to one ploidy and assigned to a different ploidy by both techniques.

Cross		Expected	Cytometry	Molecular tool	% Correct assignment
I	N	45	45	45	
	2n	20	15	15	100.0
	3n	25	25 (5)	25 (5)	100.0
II	N	56	56	56	
	2n	21	21 (2)	21 (2)	100.0
	3n	35	33	33	100.0
III	N	64	64	64	
	2n	39	39 (3)	39 (3)	100.0
	3n	25	22	19[a]	86.4
IV	N	60	60	60	
	2n	30	30	30	100.0
	3n	30	30	30	100.0
V	N	60	60	60	
	2n	30	29	29	100.0
	3n	30	30 (1)	30 (1)	100.0
Total	N	285	285	285	
	2n	140	134 (5)	134 (5)	100.0
	3n	145	140 (6)	137 (6)	97.9

[a] The three triploid individuals misclassified were detected by invert drop out pattern (see Results and discussion section).

This preferential amplification is known as drop out (Gagneux et al., 1997; Navidi et al., 1992; Taberlet et al., 1996), and chromatogram peaks corresponding with shorter alleles show higher peaks than longer ones. An inversion of this common drop out pattern would help to identify the small amount of triploids showing only two peaks. For example the Sma-USC12 locus which showed an important drop out in diploid individuals (Fig.1B) evidenced a higher intensity of the longer allele (336 allele) than in the shorter one (330 allele) at the three unassigned triploids of cross III (see the three chromatograms in Fig.1B), suggesting their triploid condition. If this correction is applied to our results, the correspondence between both techniques in this study would be 100%.

Finally, we checked the power of our technique for triploid detection in a blind sample where no parental information was available. This is a relevant point since this information is not always available at farms and requires additional analysis. The 92.9% of cold shocked individuals (26 out of 28) from the blind sample showed a three-peak genotype and were identified as triploids (see Table S2). The two unassigned individuals (3N_02 and 3N_22, see Table S2) did not show a drop out inversion at any locus, and thus, they could not be assigned as triploids. Performance of triploidy induction in our families was higher than 95% but did not reach 100% (see Section 2.1). Previous analyses of triploidy induction in turbot by cold shock showed a performance value above 95%, some individuals showing a diploid condition at most crosses (Piferrer et al., 2000). Therefore, the two individuals not assigned as triploid in the blind cross could be actually diploid according to the performance of the cold-shock technique.

4. Conclusion

Genomic resources are increasing quickly and microsatellites markers have been developed for several marine species in which triploidy can improve their culture performances. In our study, we took advantage of this information to develop an efficient molecular tool for triploid determination, based on a set of highly polymorphic and centromere-distant microsatellites. The development of molecular tools following the strategy described in this work can be applied to an important number of marine aquaculture species if suitable markers are available.

Supplementary materials related to this article can be found online at doi:10.1016/j.aquaculture.2011.11.039.

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