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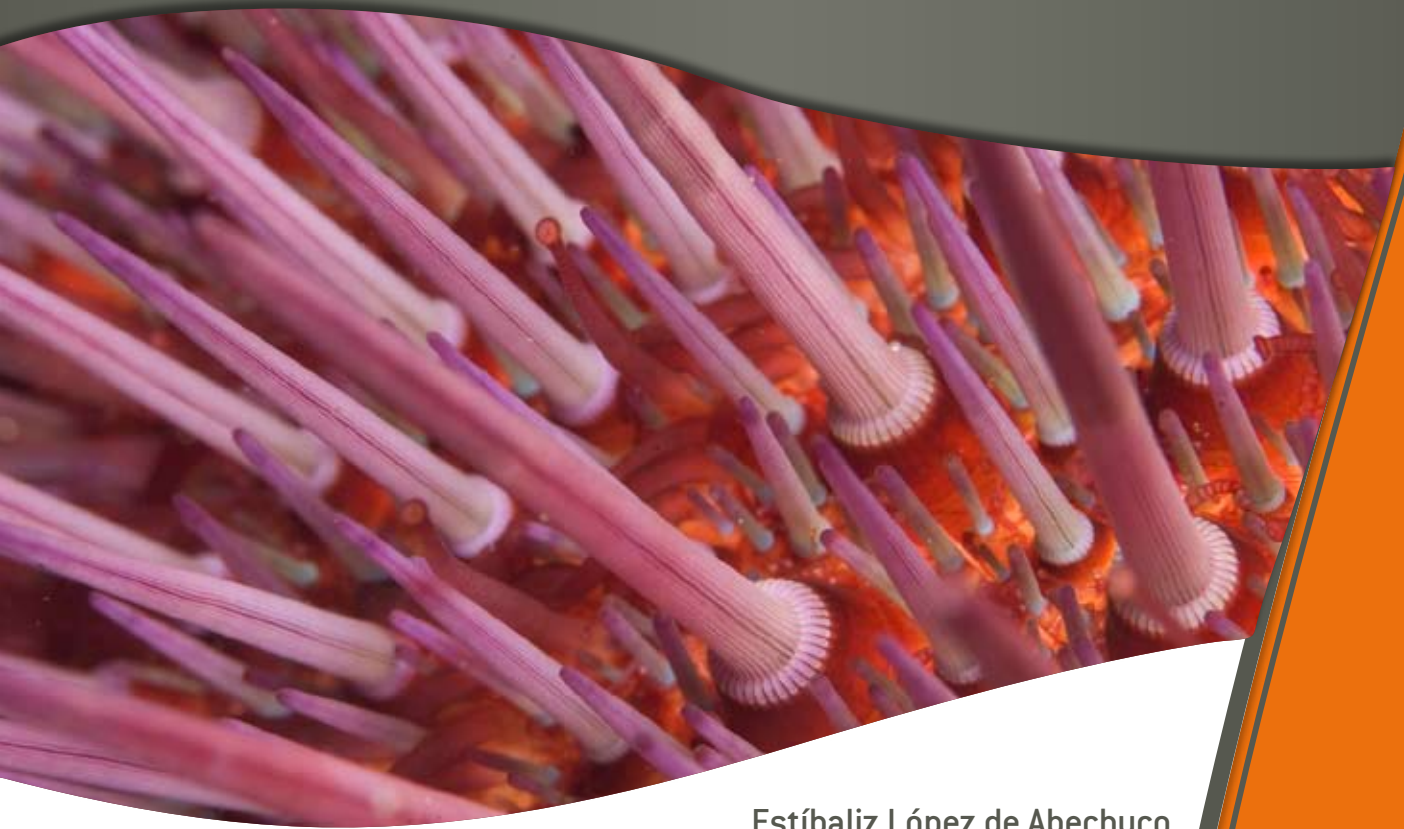

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Suitability of real-time quantitative PCR to estimate the relative telomere length in European Hake (*Merluccius merluccius* Linnaeus, 1758)

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Abstract

Telomere length measurement has been proposed as a promising tool to estimate the age of individuals in natural populations. We used real-time quantitative PCR (qPCR) to measure relative telomere length in four tissues (brain, kidney, liver and muscle) of European hake (*Merluccius merluccius*) in different groups based upon body length an otolith age estimate. We observed a high level of inter-individual differences in the measurements of relative telomere length in hakes of similar age and body length groups. The results of qPCR analysis showed a great variability in all measures and a lack of repeatability and reproducibility with significant statistical differences in the results of the different assays. The paper discusses the technical reasons for the variability in qPCR obtained in this work and by other authors.

Keywords: telomere, ageing, hake, qPCR, stock assessment.

Resumen

En este trabajo se ha realizado la puesta a punto y la estandarización de las condiciones para la PCR a tiempo real (concentración de cebadores tel1b y tel2b, diseño de cebadores para el gen de copia única, perfiles térmicos) en diferentes órganos (cerebro, músculo, hígado y riñón) de merluza europea *Merluccius merluccius*.

A continuación se diseñó una batería de ensayos en los mismos órganos procedentes de individuos con edades diferentes. El rango de tallas elegido fue de 250 mm a 750 mm, que se corresponde con una edad entre 2 y 7 años estimada a partir de la lectura de los anillos de los otolitos de los ejemplares analizados.

No se ha encontrado ninguna tendencia clara en la medida de la longitud de los telomeros en ninguno de los órganos estudiados debido a la alta variabilidad interindividual observada en individuos de la misma edad. Estas diferencias interindividuales pueden deberse a factores biológicos-genéticos o ambientales pero también a las limitaciones del método en términos de reproducibilidad que se demostró con diferencias estadísticamente significativas en los resultados de los diferentes ensayos.

Palabras clave: telomero, estima de edad, merluza, qPCR, evaluación de stocks.

Introducción

The determination of the age structure of fish commercial populations is essential for their management and conservation. However, current methods to estimate the age of important commercial fish species by means of traditional aging techniques such as otolith reading are proving not as reliable as once thought. This is especially noticeable in the European hake *Merluccius merluccius* due to the difficulty in counting and differentiating between the opaque and hyaline rings (García-Rodríguez and Esteban, 2002; Horn *et al*., 2010; Izzo, 2010; Morales-Nin and Aldebert, 1997; Piñeiro, 1997). Recent tagging studies in *M. merluccius* in the Bay of Biscay have also questioned the traditionally accepted growth rate for this species, as a result of overestimation of internationally agreed age-estimation criteria (de Pontual *et al*., 2003). Assumptions of stock dynamics based on inaccurate age estimation criteria could lead to incorrect predictions of the status of the stock and improper management advice to the fishery (Izzo *et al*., 2011b).

Studies carried out in a wide variety of eukaryotic organisms have reported that telomeres shorten with age and that this shortening could be potentially used as a marker of biological age. This relationship has been shown in humans (Harley *et al*., 1990), mammalians (Izzo *et al*., 2011a, b; McKevitt *et al*., 2002), birds (Bize *et al*., 2009; Haussmann and Vleck, 2002; Haussmann and Mauck, 2008; Haussmann *et al*., 2003a; Haussmann *et al*., 2003b; Vleck *et al*., 2003, Horn *et al*., 2011) and reptiles (Hatase *et al*., 2008; Scott *et al*., 2006). In fishes, there is no agreement, as some studies find a significant relationship between telomere shortening and age

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(Hartmann *et al*., 2009; Hatakeyama *et al*., 2008; McChesney *et al*., 2005). whereas others do not (Horn *et al*., 2010; Izzo, 2010).

Telomeres are complexes of short tandem repeated sequences of non-coding DNA found at the end of eukaryotic chromosomes. Telomeres consist of a variable number of a short G-rich evolutionary conserved DNA sequence $(TTAGGG)_n$, (de Lange *et al*., 1990; Hartmann *et al*., 2009; Haussmann and Vleck, 2002; Klapper *et al*., 1998; Meyne *et al*., 1989). The main function of telomeres is to maintain the structural stability of chromosomes protecting them from degradation and fusion events (Aubert and Lansdorp, 2008; Blackburn, 1990; Blasco, 2002) and to control of replicative senescence (Grabowski *et al*., 2005).

During the normal cell cycle the enzyme DNA polymerase is unable to replicate completely the end of the telomere, resulting in the loss of base pairs and telomere shortening (Chan and Blackburn, 2003). This loss is compensated by the enzyme telomerase that consists of an RNA subunit (TERC, Telomerase RNA Component) which acts as a template and a catalytic protein subunit (TERT, Telomerase Reverse Transcriptase) that catalyzes the elongation of telomeres (Blackburn, 1990). The end-replication problem and the lack of telomerase activity are not the only factors that contribute to telomere shortening, heritable components or oxidative stress may also contribute to telomere loss (von Zglinicki, 2002).

In fishes, we still lack the basic knowledge of how telomere length is regulated (Hartmann *et al.* 2009). Nevertheless, some studies have determined telomerase activity in different tissues of rainbow trout (*Oncorhynchus mykiss*), channel catfish (*Ictalurus punctatus*), zebrafish (*Danio rerio*) or Japanese medaka (*Oryzias latipes*) as an indirect way to quantify aging and cell proliferation of these tissues (McChesney *et al*., 2005). Other studies have focused on the localization of repetitive telomeric sequences in different fish species to study chromosome evolution and speciation several species of sturgeon (Fontana *et al*., 1998), selachian (Rocco *et al*., 2001) *Mugilidae* (Gornung *et al*., 2004) or Atlantic salmon *Salmo salar* (Perez *et al*., 1999). Very recently, some studies have explored the relationship between age and telomere dynamics in the European sea bass, *Dicentrarchus labrax*, (Horn *et al*., 2008), *O. latipes* (Au *et al*., 2009; Hatakeyama *et al*., 2008), Killifish (*Nothobranchius furzeri*) (Hartmann *et al*., 2009), flathead (*Platycephalus bassensis*), snapper (*Chrysophrys auratus*) or golden perch (*Macquaria ambigua*) (Izzo, 2010).

Knowledge of age in important commercial fish is necessary to improve information related to growth, reproduction, and overall stock health. More reliable age estimates would also allow more accurate calculations of a population's age structure, and provide information of whether the population is increasing, stable or declining.

Several experimental procedures have been developed to measure telomere length. Some of the most widely used methods like Telomere Restriction Fragments analysis (TRF) (Harley *et al*., 1990), fluorescence in situ Hybridization methods Q-FISH (Zijlmans *et al*., 1997) or Flow-FISH (Rufer *et al*., 1998) are challenging, expensive and time-consuming (Callicott and Womack, 2006). In the last decade new techniques, including Single Telomere Length Analysis (STELA) (Baird *et al*., 2003), Telomeric-Oligonucleotide Ligation Assay (T-OLA) (Cimino-Reale *et al*., 2001) and Telomere measurement by quantitative PCR (qPCR) (Cawthon, 2002) have been developed to solve these drawbacks.

In this work we have adapted the qPCR method to measure telomere length in European hake (*Merluccius merluccius*). The major advantages of this technique are that it is relatively simple, fast, less costly and highly sensitive being able to measure telomere length from very small amounts of DNA (Nakagawa *et al*., 2004). Since the publication of Cawthon's technique in 2002 it has proven its usefulness in many studies carried out in the field of biomedicine and epidemiology (Epel *et al*., 2004; O'Sullivan *et al*., 2006; Zhang *et al*., 2007). Recently, some authors have attempted to use this approach in ecological and evolutionary studies in turtles (Hatase *et al*., 2008), birds (Bize *et al*., 2009; Criscuolo *et al*., 2009) and fishes (Hartmann *et al*., 2009).

The main objective of the present work was to test whether the measure of telomere length by qPCR is a suitable technique to estimate the individual age and population age structure in different tissue samples of wild *M. merluccius*.

Materials and methods

Sample collection

Female specimens (n=15) of *M. merluccius* were collected from commercial trawlers fishing in Bay of Biscay and Great Sole (Northeast Atlantic) in 2007 and 2008. Length, total weight, gutted weight and estimated age according to otolith reading of the selected specimens are shown in Table 1. Specimens ranged in age from 2 to 8 years according to otolith reading and Age-Length Keys used by the International Council for the Exploration of the Seas (ICES) in the Divisions VIII a, b, c and d and VII (ICES, 2007, 2008). Because larger fish came from commercial boats without gonads we could not determine the gender of two individuals. Sagittal otoliths were also removed and stored in dry condition.

Table 1. Summary of the biological data of the samples used in the assays and the groups of age classes. F: Female; Undet.: Undetermined.

	Total length (cm)	Estimated age (years)	Sex
age class 1	26.5	2	F
	26.7	2	F
	27.6	2	F
	27.9	2	F
	35.8	3	F
age class 2	42.3	4	F
	43.6	4	F
	44.3	4	F
	45.3	4	F
	55.1	5	F
age class 3	62.3	6	F
	64.0	6	F
	64.1	6	F
	65.0	6	Undet.
	75.4	7-8	Undet.

DNA extraction

Tissue samples (brain, muscle, liver and kidney) from each individual were collected and stored in 1.5 ml eppendorf tubes at -20ºC until DNA extraction. Genomic DNA from all tissues was extracted using the Wizard Genomic DNA Purification kit (*Promega* Corporation, Madison, WI, USA). The integrity of DNA was checked by gel electrophoresis in 0.8 % agarose D-1 Medium EEO (Pronadisa, Conda Laboratory, Madrid, Spain) stained with Gel-Red (Biotium, Hayward, CA, USA), and after this the DNA was stored at -20ºC until its use.

Telomere length measurement by quantitative PCR

Telomere length measurement was carried out according to the qPCR method developed by Cawthon (2002) with some modifications in the primer design and concentration. The original primers designed by Cawthon were changed to have a similar GC content and closer melting temperatures (Cawthon, 2006; Gil and Coetzer, 2004).

As with the first designed primers, the optimized ones contained a mismatch every six bases in the 3' to the 5' direction. Since the last five bases of the 3'-end match perfectly to the target sequence, the DNA polymerase can synthesize the complementary strand to the telomeric sequences. The primer sequences were as follows:

te11b; 5'-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3';

te12b; 5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3' (Callicott and Womack, 2006; Gil and Coetzer, 2004).

The telomere reaction contained 12.5 µl SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK), 250 nM of the tel1b and 200 nM of the tel 2b, 1 ng genomic DNA, and enough double-distilled water to yield a 25 µl reaction.

The selected single copy gene to normalize the telomere PCR was the rhodopsin gene since this is one of the few sequences that has been obtained from hakes (GenBank accession no. EU492240, (Noren *et al*., 2008)). The rhodopsin gene is considered a single-copy gene that encodes proteins in the photoreceptors of the eye and only in tetraploid fish such as salmonids, catastomids and the carp *Cyprinus carpio* more than one copy has been found (Bailey *et al*., 1978; Larhammar and Risinger, 1994). A pair of gene specific primers and a gene specific probe were designed with Primer Express software (Applied Biosystems, Foster City, CA) to amplify an 85 bp amplicon. Forward and reverse primers for the hake rhodopsin gene were Rhod-F 5'-CGTGGCCTGGTACATCTTCA-3' and Rhod-R 5'-ACGCCGGCAGGGTCAT-3'. The TaqMan probe was 5'-CACCAGGGCAGCATATTCGGACCC-3'. For the rhodopsin PCR, we added 12.5 µl TaqMan Master Mix (Applied Biosystems), 300 nM of the Rhod-F and 250 nM of the Rhod-R, 150 mM of rhodopsin TaqMan probe, 1 ng genomic DNA, and enough double-distilled water to yield a 25-µl reaction. All samples were run in an Applied Biosystems 7000 Real-Time PCR System (Applied Biosystems, Foster City, CA). Thermocycler conditions for telomere qPCR were set at 95°C for 10 min followed by 25 cycles of data collection at 95°C for 15 s, with 54°C annealing for 2 min. The rhodopsin gene cycling conditions were 95°C for 10 min followed by 40 cycles at 95°C for 15 s, with 54°C annealing for 2 min.

A primer optimization matrix and an adjustment of the thermal cycling profile were performed for both reactions to obtain the most adequate concentration for each reagent and to establish the setting of each reaction selecting the ones that gave the lowest background in the minimum Ct.

Each plate contained dilutions of five DNA concentrations (ranging from 10 ng to 0.01 ng) of tissues from the same reference hake. Each DNA sample and negative controls were analysed in quadruplicate, and specific DNA samples were analysed in the same wells for telomere and rhodopsin reactions.

Results of each run were analysed using an ABI PRISM 7000 SDS Software 1.0 (Applied Biosystems, Foster City, USA). The Ct values (the fractional cycle number at which the well's accumulating fluorescence crosses a set threshold that is several standard deviations above baseline fluorescence) of the telomere and the single copy gene were calculated as the mean of the four aliquots of each tissue sample and sampling group.

According to the qPCR equipment specifications if the mean of the C_t obtained for each sample had a standard deviation higher than 0.38, the aliquot of which Ct value increased the deviation of the mean obtained for each sample was rejected and the average recalculated. If there were more than two Ct values that deviated from the mean, then the entire qPCR plate was redone in order to minimize error in the assay.

For each tissue, analysis regression lines for PCR (telomere and rhodopsin gene) were compared using the serial dilution of the reference sample. From the value of the slope, PCR efficiency was calculated according to the equation $E=(10^{(-1/slope)} - 1) \times 100$ where E is the PCR efficiency, and S is the slope of the standard curve. For the telomere PCR reaction the obtained efficiency ranged from 89 and 113 % and for the rhodopsin gene reaction it was between 91 to 99 %.

In order to know the factor by which a DNA sample differs from a reference DNA in its ratio of telomere repeat copy number (T) to a single copy gene number (S), T/S ratio was calculated according to the following formula: $T/S = 2^{-iCt}(2^{-iCtaverage})^{-1}$;

iC_t = increment of C_t

where $iC_t = C_t^{\ rhodopsin} - C_t^{\ telomere}$

and $iC_t^{average}$ is the average of all the iC_t obtained in the plate.

The assay for each tissue and age class was performed on three different days under the same experimental conditions and placing the samples always in the same wells in each repetition and using four DNA replicates of each tissue.

Otolith preparation and reading

The otolith preparation and age determination were in accordance with the methodology described in the EU BIOSDEF (Anon., 1998) and DEMASSESS international projects (Anon., 2000). Each otolith was mounted in a black resin block and cut in a section of 0.5 mm. Sections were mounted on glass slides and read in a stereomicroscope. Otolith interpretation started from the nucleus towards the edge and only the hyaline rings were counted.

Rings were counted twice by two readers trained according to the experience obtained from international workshops on hake ageing studies.

Statistical analysis

Fisher's Least Significant Difference (LSD) and Mann-Whitney (Wilcoxon) tests were used to determine significant differences between T/S ratio obtained in eight DNA replicates of each tissue grouped in three different age classes (Table 1). For these analyses Statgraphics Plus 5.0 (Statpoint Technologies, Inc., Virginia, USA) and Microsoft Office Excel 2003 (Microsoft Corporation, USA) computing and graphics software were used.

Results

Regression analysis was used to examine the relations between the dependent variables of telomere shortening (T/S ratio), and the age of hakes estimated by otolith reading and the independent variable of length size in each of the four tissues analysed (Figure 1). The ratio obtained for each samples is a result of the medium value obtained after performing the assay on three different days.

The coefficient of determination (R^2) between the estimated age by otoliths and length size of hakes was higher than 0.98 indicating the increase of age estimated by the number of rings in the can be explained by the increase of the length of hakes.

It was observed high variability between the results of three qPCR replicas in all tissues analysed. Thus, in some samples the coefficient of variation of T/S ratio replica reached values higher as 31% in brain and muscle, 38% in kidney and 53% in liver.

Discussion

Many fish species, unlike mammals, show indeterminate growth coupled with slow senescence (Reznick *et al*., 2002; Woodhead, 1998) which makes the age estimation in these species very difficult.

The variability of the qPCR method (Criscuolo *et al*., 2009; Hatase *et al*., 2008; Shen *et al*., 2007) didn´t allow estimating telomere length, and therefore we could not confirm nor reject correlation between age and relative telomere length. In the present study to reinforce the value of the test the qPCR analysis was repeated with the same aliquots and under the same conditions in three different time series showing a relatively high variation among the results obtained in the different repetitions. This poor repeatability is in agreement with the results studies that showed a lack of significant or low correlations among different measures and considerable variability among different batches of samples (Shen *et al*., 2007).

Although many papers state the reliability of the qPCR method, the repeatability of this relative method is discussed, not often tested, reported or is relatively high (Bize *et al*., 2009; Horn *et al*., 2010; O'Callaghan *et al*., 2008; Svenson and Roos, 2009).

The qPCR it is a technique that does not provide an absolute measure of telomere length and needs the use of a single-copy-gene as a reference to estimate the ratio by which the number of telomeric repeats differ in the number of copies of a single-copy-gene. Thus, the precision of the method depends on the nucleotide sequence of single gene whose number of copies is assumed invariant under the experimental conditions (Lin and Yan, 2005).

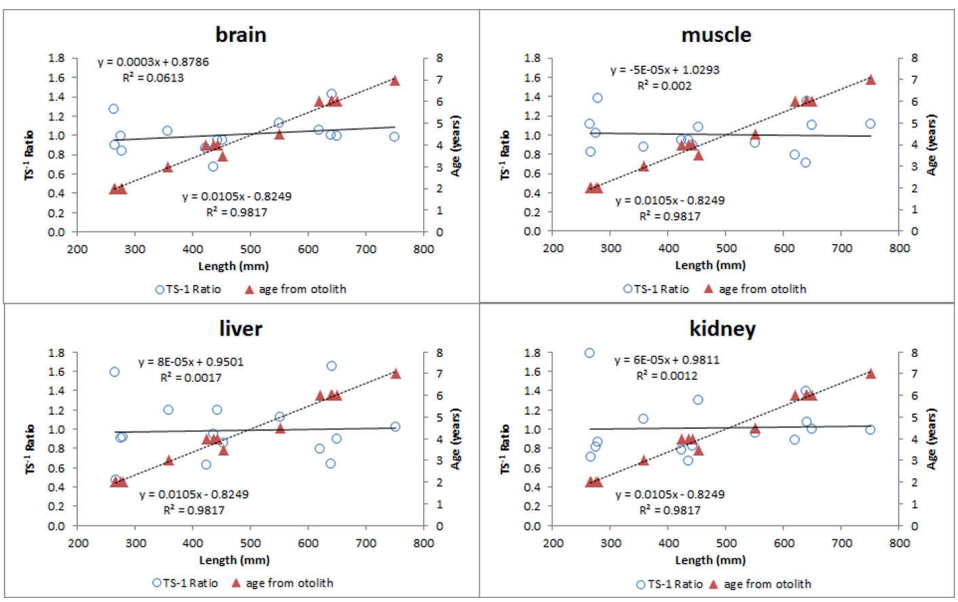

Figure 1. Comparison of measures of telomere length. Combined linear regressions between TS^{-1} ratio and Age estimated by otolith reading vs Length in four tissues of Northern hake of different sizes. Upper equation in each plot belongs to the TS^{-1} ratio vs Length regression and lower equation to Age estimated by otolith vs Length.

Besides in a qPCR analysis all telomeric sequences of different cells of a tissue sample are amplified in the same reaction. We do not know up to what point the heterogeneity of the different individual cells within the same tissue influence the results of this assay.

Some studies have also questioned the validity of the results published by other studies based upon the efficiency of the telomere reaction and the differences between the standard curve and the C_t values (Horn, 2008a; Zhang *et al*., 2007). Constant amplification efficiency is an important factor for reliable comparison between samples since small efficiency differences between two reactions can generate a false T/S ratio (Bustin *et al*., 2005; Dunshea *et al*., 2011; Pfaffl, 2001). Although the deviation between the efficiencies of the samples run in the same day was generally in an acceptable range (± 10%) (Applied_Biosystems, 2003; Stratagene, 2004), the values for this parameter obtained in different days and in the same samples showed a high variability as well.

Taken together, this suggests that the measurement of telomere length by qPCR method needs more methodological consensus and optimization to solve the methodological difficulties in telomere measures.

O'Callaghan *et al*. (2008) modified the qPCR method designed by Cawthon introducing a standard oligomer to obtain absolute measures of telomere lengths. This new approach provides more reproducible data than the relative method. Nonetheless, the use of this Absolute qPCR in Australian sea lions showed a coefficient of variation and a measure of precision of 47.16 % and 33.35 % respectively (Izzo *et al*., 2011b).

Recently, a new method developed by (Cawthon, 2009) has improved the reliability of the qPCR telomere assay. This improvement increases the correlation between the measure obtained by means of a multiplex qPCR and the TRF length measured by Southern blot.

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