Sex identification of Albacore using a low cost genetic method

Helary Louise[1], Chevrier Thomas[1], Roumagnac Marie[1], Chanut Jérémie[2], Nieblas Anne Elise[2], Dominique Cowart[2], Padron Mariana[1,3], Brisset Blandine[1], Evano Hugues[1], Bernard Serge[4], Kerzerho Vincent[4], Rouyer Tristan[5], Bonhommeau Sylvain[1]

[1] IFREMER, DOI, La Réunion, France
[2] Company for Open Ocean Observations and Logging, La Réunion, France
[3] Versailles, France
[4] LIRMM/CNRS - Université de Montpellier, France
[5] MARBEC/IFREMER, Sète, France

Corresponding authors
louise.helary@ifremer.fr
thomas.chevrier@ifremer.fr
sylvain.bonhommeau@ifremer.fr

Abstract

Sex identification of animal species is a critical piece of information to derive parameters for population dynamic models. In the context of stock assessment (SA) for marine population, sex identification provides information about the sex-ratio of the population which is subsequently used to calculate the stock spawning biomass. In these SA models, sex-ratio can be set to a constant value throughout the lives of individuals (e.g. 0.5) or age-structured to account for changes linked to the physiology of individuals (e.g. females may live longer and represent a larger proportion of the population) or the selectivity of the fishery (e.g. a gender may be more accessible to the fishery at specific stages). The most common methods to identify sex are derived from direct observation of gonads. However, scientists must access the whole fish, which is rarely the case for large pelagic species in the IOTC fishery as fish that are landed have been gutted. Here we show the preliminary results of sex identification of albacore (*Thunnus alalunga)* using a genetic method: Amplified Fragment Length Polymorphism (AFLP). This polymerase chain reaction (PCR)-based genetic tool is a highly sensitive method for detecting polymorphisms in DNA. We tested the hypothesis that male and female albacore present a genetic polymorphism linked to sex. Using a multiplexing technique (i.e. a combination of different restriction enzymes and PCR primers), we identified potential locations in the albacore genome where polymorphism could occur. Comparing results between 3 males and 3 females of albacore, we were able to identify 1 marker over 64 combinations of primers that led to potential sex-specific polymorphism identification. Higher sampling (40 males and 40 females) will be performed to confirm these results. This method has the advantage of being low cost, simple to develop, requires few genetic laboratory analysis or preparation (DNA extraction, PCR, electrophoresis and a capillary sequencer for DNA fragment analysis) and the genetic material required is minimal and can be taken from a living or dead animal.

Introduction

Sex is a fundamental biological parameter used in stock assessments. Sex determination and differentiation in fishes are highly variable processes, with genetic, environmental (e.g. temperature, pH), and even social factors having a strong influence on them (Devlin and Nagahama, 2002; Guerrero-Estévez and Moreno-Mendoza, 2010). The ability to correctly identify sex is an important and sometimes complex task, particularly for species that lack clear sexual dimorphism or sex-specific characters. Sex determination is most of the time impossible at landings for IOTC fisheries as fish are gutted onboard for preservation purposes.

For stock assessment modeling, sex is a critical information as it is used to estimate the population sex-ratio. For an aged-structured model, sex-ratio combined with fecundity at age or stock-recruitment relationship define the renewal capacity of the population. This source of information about sex has hence critical impacts on the stock assessment projections.

The identification of sex-linked DNA markers is key genetic information allowing the development of molecular sexing tools (Gamble and Zarkower, 2014; Lai et al., 2014; Robertson and Gemmell, 2006). This approach is non-lethal and offers an opportunity to study endangered species, and/or to work in situations (e.g. mark-recapture programmes and/or experiments) where the sex of living individuals needs to be determined.

Even though most fish species do not possess heteromorphic sex chromosomes, genetic sex determination has been found in a wide variety of fish species using alternative techniques to those based on karyotyping. Using the Amplified Fragment Length Polymorphism (AFLP) technique, Agawa et al., (2014) identified one male-specific marker in the Pacific Bluefin tuna (*Thunnus orientalis*). Sex-linked AFLP markers were also isolated from the three-spined stickleback *Gasterosteus aculeatus* (Griffiths et al., 2000), and the rainbow trout *Oncorhynchus mykiss* (Felip et al., 2005). A sex-linked microsatellite marker was identified in the nine-spined stickleback *Pungitius pungitius* (Shikano et al., 2011) and Chen et al., (2007), successfully isolated seven female-specific AFLP markers in the half-smooth tongue sole, *Cynoglossus semilaevis*. However, the variability in the number and type of

loci involved in fish sex determination implies that sex-linked genetic markers are typically species- or population-specific.

Albacore, *Thunnus alalunga* is one of the most target species of the commercial tuna fishery (IC Chen et al. 2005) and it is managed internationally by the International Commission for the Conservation of Atlantic Tunas (ICCAT), Indian Ocean Tuna Commission (IOTC), and the Inter-American Tropical Tuna Commission (IATTC). Given its economic importance and in order to help elucidate genetic sex determination mechanisms in the species, the aim of this study was to identify sex-specific markers in *T. alalunga* based on cDNA samples from known male and female individuals, using the Amplified Fragment Length Polymorphism (AFLP) technique (Vos et al., 1995).

Material and Method

Biological samples

Albacore individuals of various lengths (LJFL: 78 cm to 99 cm) were obtained from commercial fisheries (tuna pole, purse seine and longline) in the Indian Ocean (Figure 1). A total of 6 samples from 3 males and 3 females were used (Table 1).

Table 1 : Samples informations

Sex	ID	Name	FL (cm)	[DNA] ng/µL	Location
Male	B1_158	M8	89	60.4	Seychelles
	B1_156	M9	92	53.8	Seychelles
	C1_029	M10	81	103	South Africa
Female	A1_026	F8	99	71.6	Reunion
	C1_078	F9	78	240	South Africa
	C1_058	F10	80	124	South Africa

Phenotypic sex was determined by examining gonadal morphology. Muscle tissues were collected and preserved in Ethanol (95%), and stored at -20 °C.

Genetic analyses

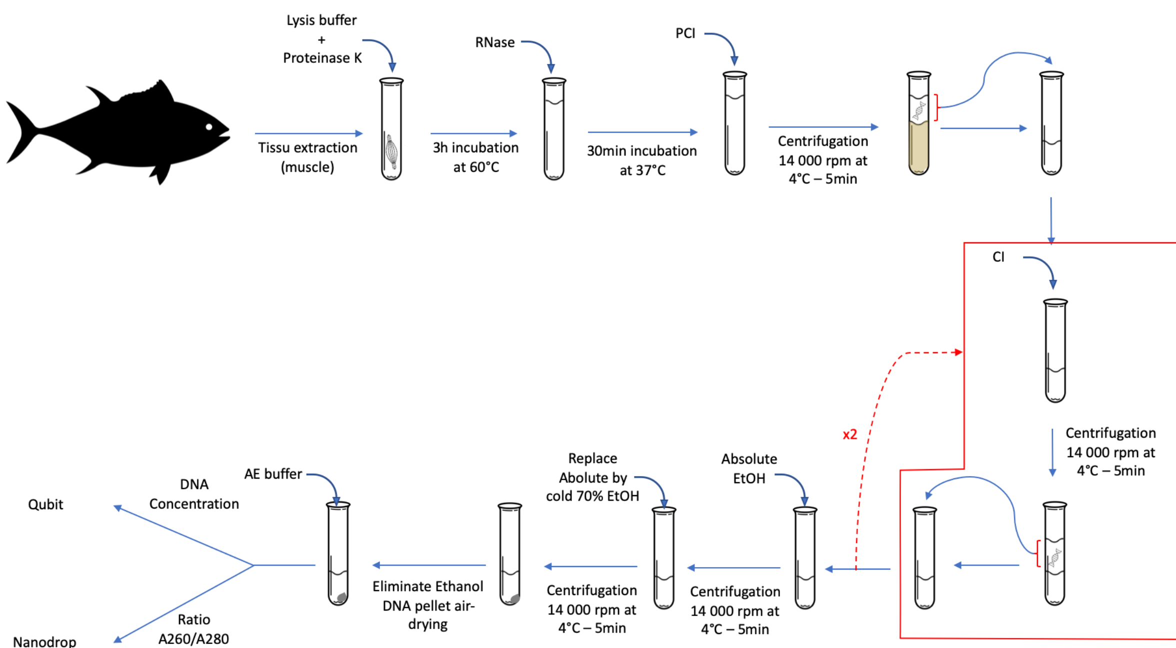
- **DNA extraction**

DNA extraction from 30±2 mg of muscle was performed using a Phenol/Chloroform/Isoamyl alcohol (PCI) method. It's based on a liquid-liquid DNA isolation and allows separating the nucleic acids (DNA and RNA) to the proteins thanks to their different solubility. Before that, a chemical lysis was induced using a lysis buffer and Proteinase K (NaCl 120mM, Tris-HCl pH8 10 mM, EDTA 10 mM, Urée 4M and SDS 5%) for 3h at 60°C.

RNase (4 mg/mL, Qiagen) was then added for 30 min at 37°C to eliminate RNA. PCI was added and centrifuged (14 000 rpm at 4°C, 5 min). The aqueous supernatant phase was recovered

and chloroform was added and centrifuged at the same conditions as before. This step was realized twice. To precipitate DNA, absolute ethanol was incorporated. Another centrifugation was applied to the solution to put down all nucleic acid and create a DNA pellet. The solution was eliminated and 70% ethanol was added to wash the pellet. After removing the 70% ethanol and air-drying the pellet, DNA was eluted in buffer AE (Qiagen). All steps of DNA extraction by the PCI method are shown in Figure 2.

Figure 2: DNA extraction using PCI method



The extracted DNA's concentration was measured using an Invitrogen™ Qubit™ 4 Fluorometer (Waltham, Massachusetts, États-Unis) and quality ratio A260/280 was obtained by using NanoDrop™ Lite Spectrophotometer. All DNA samples were then diluted to 10 ng/µL.

- **AFLP technique**

AFLP analysis was carried out as described by Vos et al., 1995 with minor modifications. DNA samples were digested with EcoRI and MseI restriction enzymes (Takara Bio Inc.). Restriction

enzymes are enzymes that cut, or digests, DNA at specific sites (restriction site). Recognition sequences in DNA differ for each restriction enzyme, producing differences in length, sequence and strand orientation (5' end or 3' end). Here two restriction enzymes were used: a rare cutter (EcoRI) and a frequent one (MseI). The sequences recognized by EcoRI and MseI are presented in Table 2.

Table 2: Restriction enzymes and their restriction sites according to DNA strand orientation.

Restriction enzyme	Restriction site
EcoRI	5'... G A A T T C ...3' 3'... C T T A A G ...5'
MseI	5'... T T A A ...3' 3'... A A T T ...5'

DNA digestion was performed at the same time as adapter ligation. The EcoRI and MseI adapters (Table 3) were ligated to the DNA fragments to generate template DNA for amplifications, using T4 DNA ligase (Takara Bio Inc.) during 2 hours at 37°C.

Table 3: Adaptators sequences

Enzyme	Adaptors	Sequence (5' —>3')
EcoRI	EcoRI-A1	CTC GTA GAC TGC GTA CC
	EcoRI-A2	AAT TGG TAC GCA GTC TAC
MseI	MseI-A1	GAC GAT GAG TCC TGA G
	MseI-A2	TAC TCA GGA CTC AT

Once the ligation was completed, two DNA amplifications were done, the pre-selective and the selective amplification. In the pre-selective amplification step, adapter common primers were used (Table 4). Polymerase Chain Reaction (PCR) amplifications were performed for 20 cycles with the following cycle profile: a 30s DNA denaturation step at 94 °C, a 30s annealing step at 56 °C and a 1 min extension step at 72 °C. Pre-selective amplification allows a few fragments to be selectively

amplified.

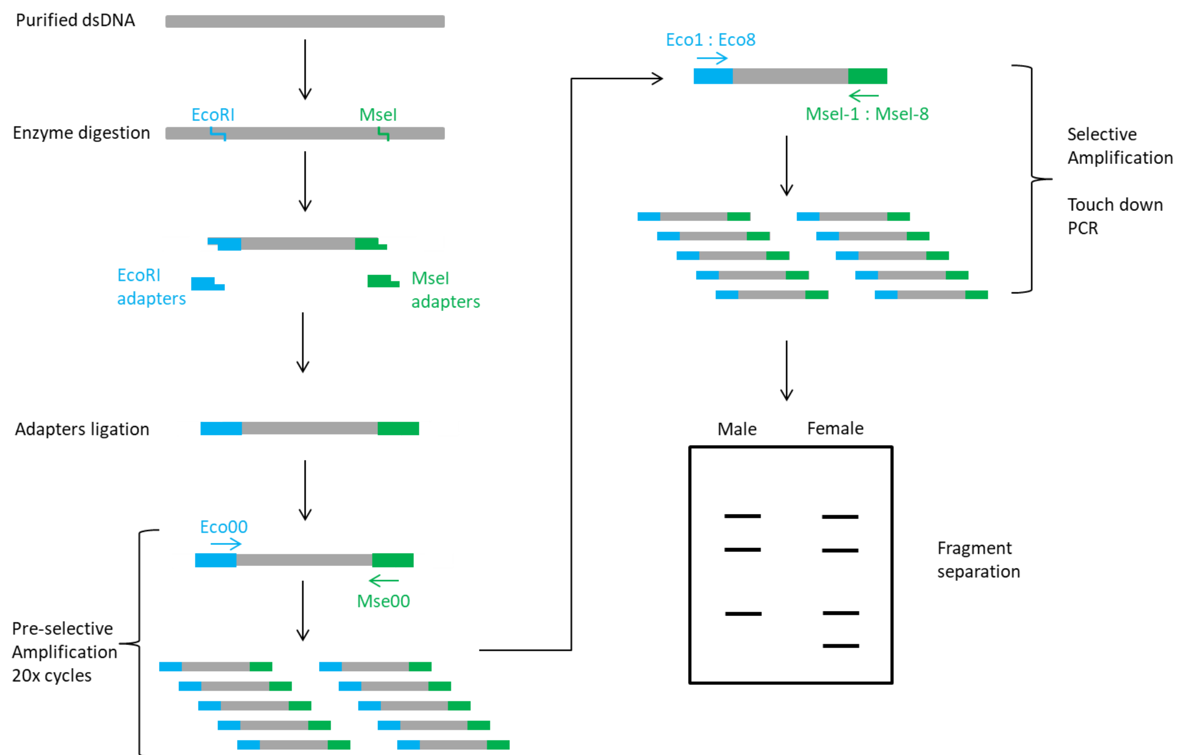
	Oligo sequence (5' to 3')	Oligo name	5 ' Mod
Pre-selective primers	GAT GAG TCC TGA GTA A	Mse00	
	GAC TGC GTA CCA ATT C	Eco00	
Selective primers	GAC TGC GTA CCA ATT C AAC	Eco1	6-FAM
	GAC TGC GTA CCA ATT C AAG	Eco2	Yakima Yellow
	GAC TGC GTA CCA ATT C ACA	Eco3	DragonFly Orange
	GAC TGC GTA CCA ATT C ACT	Eco4	ATTO 565
	GAC TGC GTA CCA ATT C AGA	Eco5	6-FAM
	GAC TGC GTA CCA ATT C AGC	Eco6	Yakima Yellow
	GAC TGC GTA CCA ATT C ATC	Eco7	DragonFly Orange
	GAC TGC GTA CCA ATT C ATG	Eco8	ATTO 565
	GAT GAG TCC TGA GTA A CAT	Mse1	
	GAT GAG TCC TGA GTA A CAA	Mse2	
	GAT GAG TCC TGA GTA A CCA	Mse3	
	GAT GAG TCC TGA GTA A CCT	Mse4	
	GAT GAG TCC TGA GTA A CTA	Mse5	
	GAT GAG TCC TGA GTA A CTT	Mse6	
	GAT GAG TCC TGA GTA A CGA	Mse7	
	GAT GAG TCC TGA GTA A CGC	Mse8	

Table 4: Primer sequences designed for AFLP

Selective amplification was performed using 64 different primers combinations from 8 EcoRI primers and 8 MseI primers (Table 4) with five-times diluted pre-selective PCR product as template. Primers were the same as pre-selective primers but with 3 extra nucleotides to the 3’ ends to reduce the number of fragments amplified by the DNA polymerase. EcoRI primers were labeled with one of the fluorescent dyes Yakima Yellow™, DragonFly Orange™, ATTO 565 or 6-FAM (Table 4). PCR amplifications were performed with the following cycle profile: 30s DNA denaturation step at 94 °C, a 30s annealing step of variable temperature and a 2 min extension step at 72 °C. The annealing temperature in the first cycle was 65 °C, and was subsequently reduced each cycle by 0.7 °C for the next 12 cycles. The profile continued with a 30s DNA denaturation step at 94 °C, a 30s annealing step

at 56 °C and a 2 min extension step at 72 °C for the next 24 cycles. Fluorescently labeled fragments for each primer combination were analyzed on an ABI 3730XL DNA sequencer by capillary electrophoresis. The resulting genotyping data were scanned in fsa files. All steps of AFLP techniques are shown in Figure 3.

Figure 3: AFLP principle



- **Electrophoresis**

Ten microliters of PCR product and 2 µL of DNA-dye Non-Tox were mixed and loaded onto a 2% agarose gel containing 8 µL of Midori Green. The electrophoresis was running in the TE buffer at 100V for 60 min. The DNA bands were observed under ultraviolet light and photographed using InfinityCapt (Vilber Lourmat Sté, France).

Data analyses

All the analyses were done with CRAN R (version 3.5.3) and the {*Fragman*} package (Covarrubias-Pazaran et al., 2016). One of the most important things to perform was to import the

correct ladder to find the real peaks corresponding to the expected weights using the *ladder.info.attach* function. In our case, it was GeneScan™ 600 LIZ™, all peaks for this ladder are presented in the appendix. All samples with a correlation for the ladder under 0.99 were removed.

Results

A total of 64 combinations of selective primers were tested resulting in thousands of scorable peaks across individuals. Over these peaks, one was of interest to differentiate males and females. It's presented below with the primer combination that allowed it to be obtained.

- **Combination Eco3 - Mse7 :**

The potential AFLP marker for *T. alalunga* was observed with the Eco3-Mse7 primers combination. Here, all samples have a correlation with ladder higher than 0.99.

For the 3 females, a peak at 23 bp is observed with an intensity higher than 1500 (Fig. 4). On the other hand, for the 3 males this peak is lower than the detection limit at 23bp (Fig. 5).

Figure 4 : Female DNA fragment intensities read at 23 bp for Eco3-Mse7

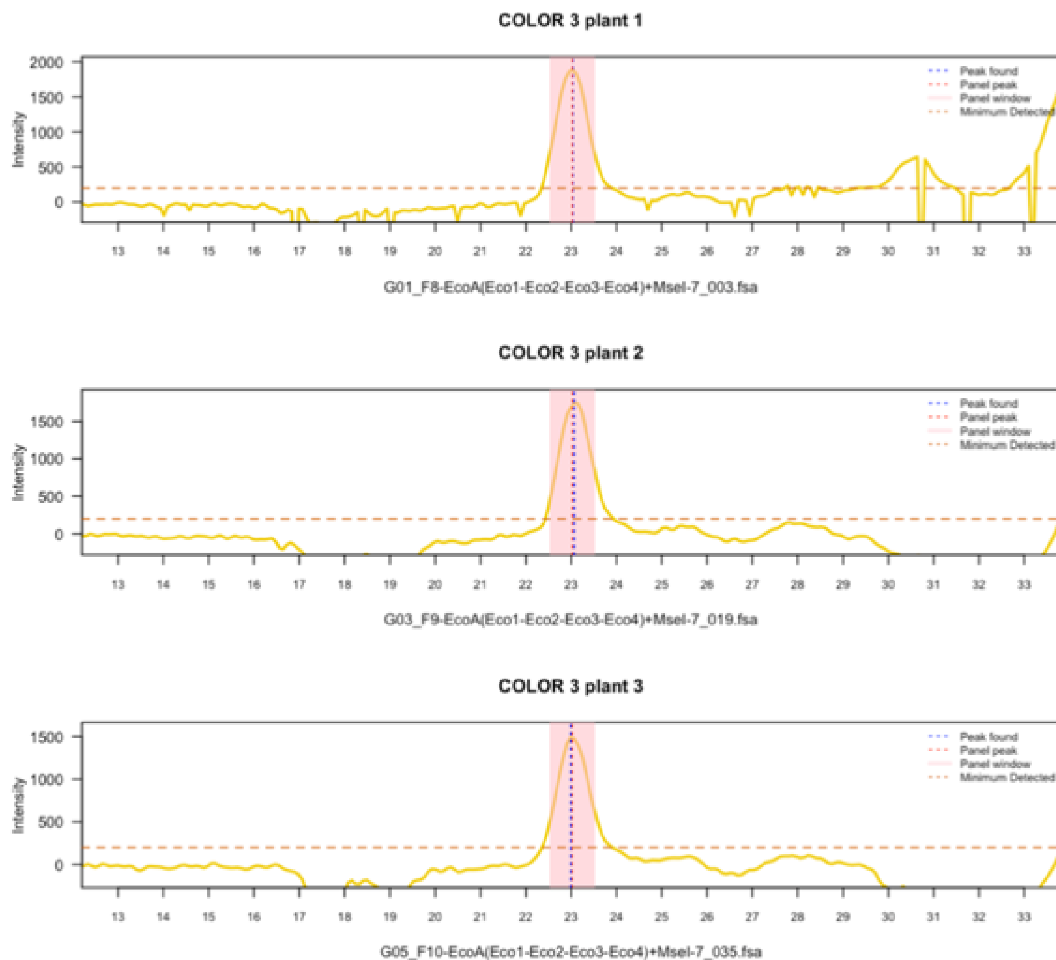
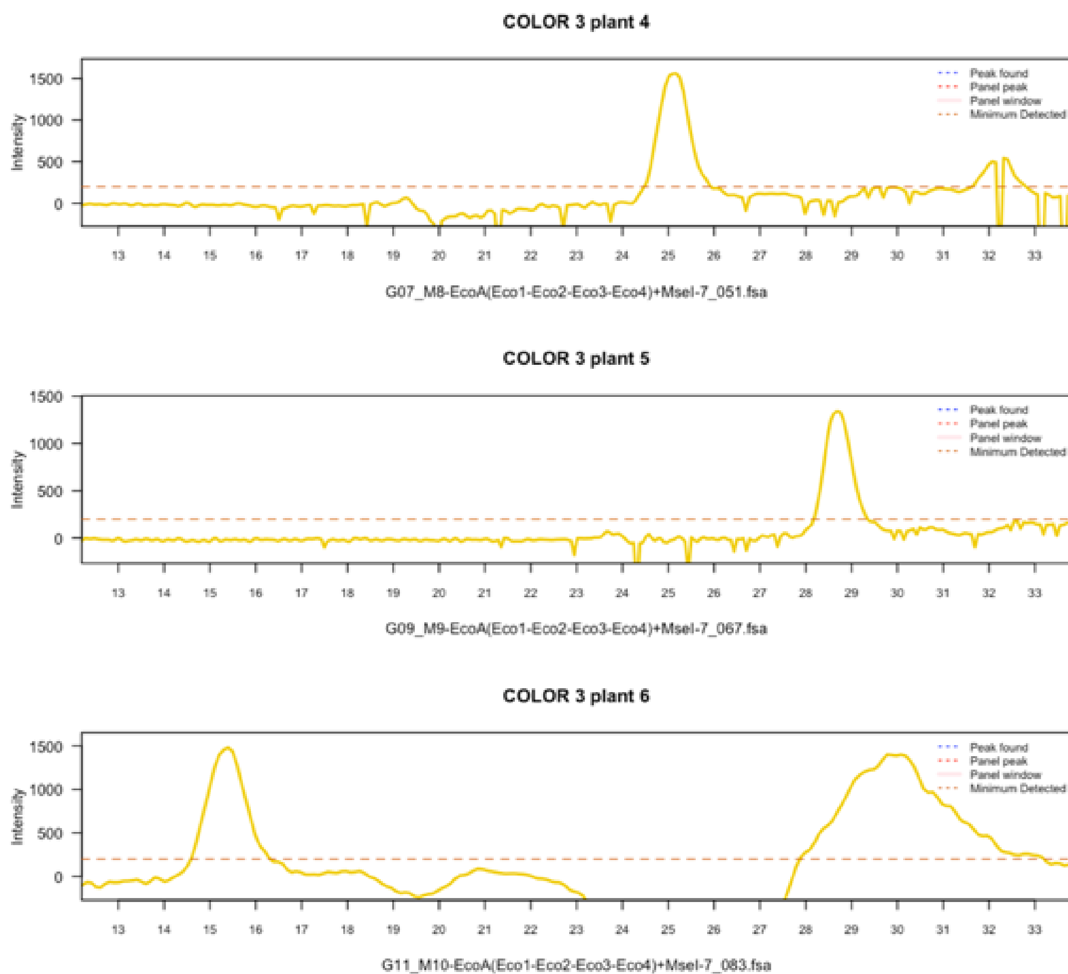


Figure 5 : Male DNA fragment intensities read at 23 bp for Eco3-Mse7



Discussion

The preliminary analyses for sex determination of albacore using a genetic method (AFLP) are very promising. We have identified a potential marker that could be used to easily identify the sex of albacore individuals. This work is a first step to investigate the potential application of AFLP for sex identification of Tunas. We plan to extend the number of samples to 40 females and 40 males to confirm the results and identify robust markers of sex.

If this genetic technique for sex identification can be validated, it can provide an efficient tool for getting sex information about individuals at landings (while fish are gutted) or on live fish (e.g. tagging) with genetic sex determination (GSD). This technique also has the advantage of being relatively low-cost (∼4,20 $ per sample from DNA extraction to electrophoresis) and to give rapid results (∼1 day from DNA extraction to electrophoresis for 96 samples depending on laboratory

equipment). This opens the opportunity for large-scale sampling programs with low lab equipment requirements.

References

Agawa, Y., Iwaki, M., Komiya, T., Honryo, T., Tamura, K., Okada, T., Yagishita, N., Kobayashi, T., Sawada, Y., 2014. Identification of male sex-linked DNA sequence of the cultured Pacific bluefin tuna *Thunnus orientalis*. Fish Sci 81, 113–121. doi:10.1371/journal.pgen.1002798

Chen, S.-L., Li, J., Deng, S.-P., Tian, Y.-S., Wang, Q.-Y., Zhuang, Z.-M., Sha, Z.-X., Xu, J.-Y., 2007. Isolation of Female-Specific AFLP Markers and Molecular Identification of Genetic Sex in Half-Smooth Tongue Sole (*Cynoglossus semilaevis*). Mar Biotechnol 9, 273–280. doi:10.1007/s10126-006-6081-x

Covarrubias-Pazaran G, Diaz-Garcia L, Schlautman B, Salazar W, Zalapa J. Fragman: An R package for fragment analysis. 2016. BMC Genetics 17(62):1-8.

Devlin, R., Nagahama, Y., 2002. Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. Aquaculture 191–364.

Felip, A., Young, W.P., Wheeler, P.A., Thorgaard, G.H., 2005. An AFLP-based approach for the identification of sex-linked markers in rainbow trout (*Oncorhynchus mykiss*). Aquaculture 247, 35–43. doi:10.1016/j.aquaculture.2005.02.014

Gamble, T., Zarkower, D., 2014. Identification of sex-specific molecular markers using restriction site-associated DNA sequencing. Molecular Ecology Resources 477. doi:10.1093/bioinformatics/btr346

Griffiths, R., Orr, K., Adam, A., Barber, I., 2000. DNA sex identification in the three-spined stickleback. J Fish Biology 57, 1331–1334. doi:10.1006/jfbi.2000.1386

Guerrero-Estévez, S., Moreno-Mendoza, N., 2010. Sexual determination and differentiation in teleost fish. Rev Fish Biol Fisheries 20, 101–121. doi:10.1139/f76-122

I-Ching, C., Pei-Fen L., Wann-Nian, T., 2005. Distribution of albacore (Thunnus alalunga) in the Indian

Ocean and its relation to environmental factors. Blackwell Publishing Ltd, Fish. Oceanogr.

Lai, X., Gao, H., Yan, B., Yan, W., Shen, S., 2014. Identification of sex-linked DNA markers by AFLP in *Portunus trituberculatus*. Gene 544, 216–219. doi:10.1016/j.gene.2014.04.034

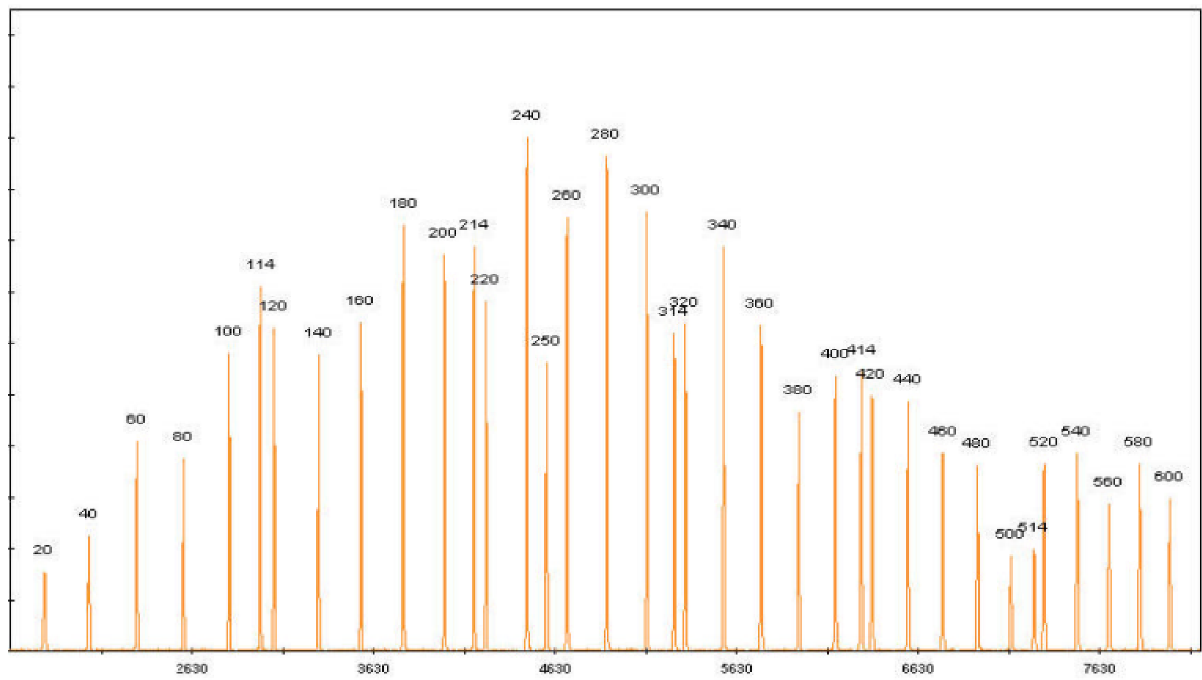
Morozova, O., & Marra, M. A. (2008). Applications of next-generation sequencing technologies in functional genomics. Genomics, 92(5), 255–264. https://doi.org/10.1016/j.ygeno.2008.07.001

Robertson, B.C., Gemmell, N.J., 2006. PCR-based sexing in conservation biology: Wrong answers from an accurate methodology? Conserv Genet 7, 267–271. doi:10.1007/s10592-005-9105-6

Shikano, T., Herczeg, G., Merilä, J., 2011. Molecular sexing and population genetic inference using a sex-linked microsatellite marker in the nine-spined stickleback (*Pungitius pungitius*). BMC Research Notes 4, 1–6. doi:10.1186/1756-0500-4-119

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., Zabeau, M., 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Research 23, 4407–4414.

Appendix



Appendix 1 : GeneScan™ – 600 LIZ® Size Standard