Sex identification of swordfish using a low cost genetic method

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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 swordfish (*Xiphias gladius*) 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 swordfish 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 swordfish genome where polymorphism could occur. Comparing results between 3 males and 3 females of swordfish, we were able to identify 2 markers 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.

Swordfish, *Xiphias gladius*, is one of the most widely distributed marine pelagic fish species worldwide. It is a highly commercial species that constitutes an important fishery resource in the Pacific and Indian Ocean, 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).

Sex determination markers have not been yet identified for this species, and swordfish individuals do not exhibit morphological characters enabling to distinguish males from females, although females do tend to be larger (Palko et al., 1981). 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 *X. gladius* 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

Swordfish individuals of various lengths (LJFL: 50 cm to 350 cm) were obtained from commercial fisheries in the Indian Ocean (Table A1). A total of 6 individuals of known sex from 2 different locations was used, 4 individuals (2 females named F5 and F7 and 2 males named M6 and M7) from the island of La Réunion, and another 2 individuals (1 female named F6 and 1 male named M5) from the Gulf of Bengal.

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 is 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 (NaCl 120mM, Tris-HCl pH8 10 mM, EDTA 10 mM, Urée 4M and SDS 5%) for 3h at 60°C. It attacks the lipid layers and membranes of the samples and initiate protein denaturation:

- **NaCl** prevents DNA denaturation.

- **Tris-HCl** plays a role in maintaining the pH of the solution and will permeabilize membrane cells.

- **EDTA** is a chelating agent that blocks enzymatic activities such as DNases.

- **SDS** is an anionic detergent that assists in the denaturation of protein membrane cells.

- **Proteinase K** is an enzyme (endopeptidase), which digests proteins, extracts nucleic acids (DNA or RNA) and removes contaminants from the nucleic acid preparation. It inactivates DNases and RNases. And finally, its activity is stimulated by denaturing agents such as SDS.

RNase (4 mg/mL, Qiagen) was then added 30 min at 37°C to destroy 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 1.

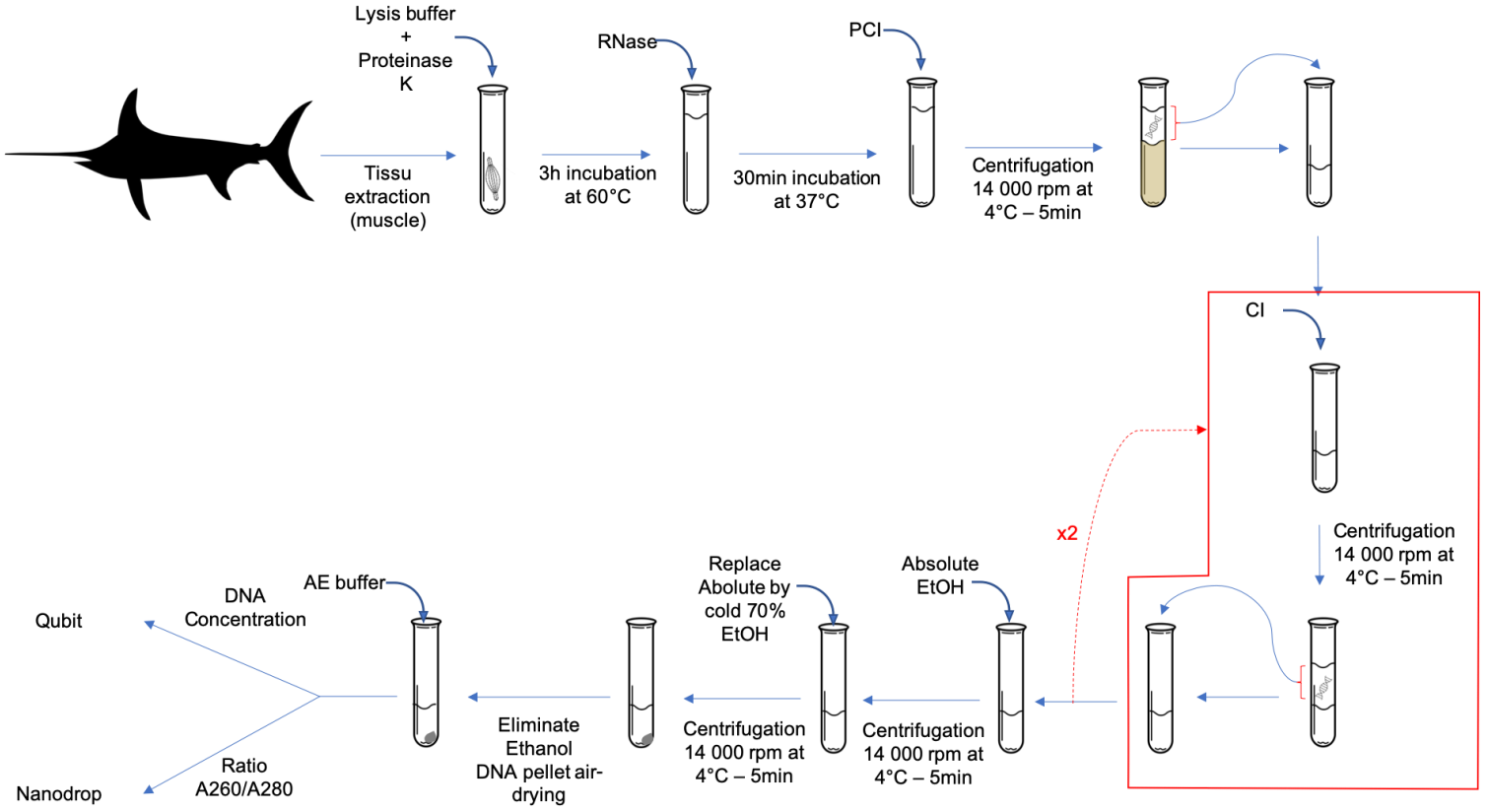


Figure 1: DNA extraction using PCI method

DNA was quantified using Qubit™ dsDNA BR assay kit 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 1.

Table 1 : Restriction enzymes and their restriction sites (arrows) 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 2) 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 2 : Adaptators sequences

Adaptator's name	Sequence (5' → 3')
EcoRI-A1	CTC GTA GAC TGC GTA CC
EcoRI-A2	AAT TGG TAC GCA GTC TAC
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 3). 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

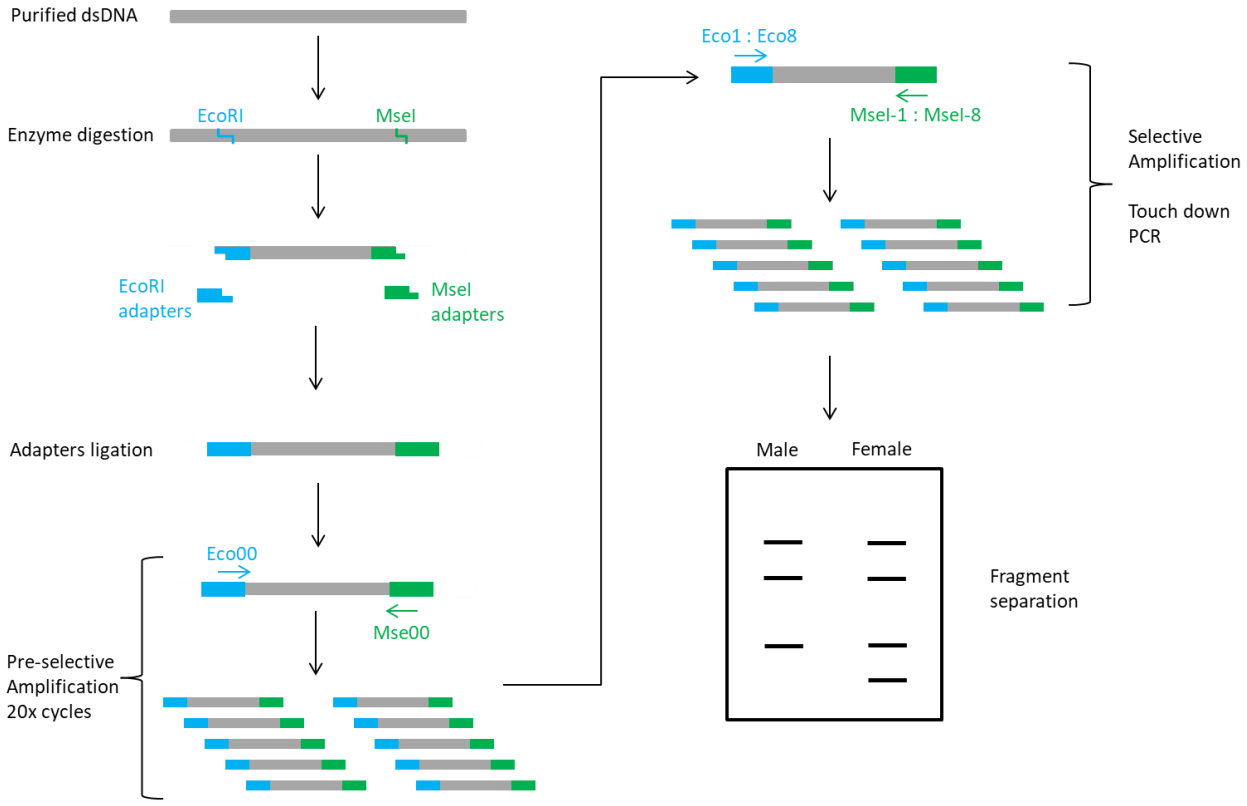
72 °C. Pre-selective amplification allows a few fragments to be selectively amplified.

Table 3: Primer sequences designed for AFLP

	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	

Selective amplification was performed using 64 different primers combinations from 8 EcoRI primers and 8 MseI primers (table 3) 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 labelled with one of the fluorescent dyes Yakima Yellow™, DragonFly Orange™, ATTO 565 or 6-FAM (Table 3). 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 was

continued with a 30 s 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 labelled fragments for each primer combination were analysed 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 2.

Figure 2 : AFLP principle

- Electrophoresis

At the end of each amplification, a small DNA portion was put into a 2% agarose gel for electrophoresis analysis to check if PCR was correctly achieved (Fig. 2). Gel electrophoresis is a method used to separate macromolecules like DNA, RNA or proteins by applying an electric field to move the molecules through an agarose matrix to the positive charge. DNA fragments, globally negatively charged,

migrate more or less far in the agarose gel matrix depending on their size. A DNA molecular weight marker (SmartLadder, Eurogentec) was added in the agarose gel to identify migrated DNA fragments according to their size. A DNA intercalant (Midori) was incorporated in the agarose gel in order to visualize the different DNA bands by UV exposure once migration completed.

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 (Fig. A2). 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, three were of interest to differentiate males and females. They are presented below with the primers combination that allowed them to be obtained.

- **Combination Eco4 - Mse2:**

The first potential AFLP marker for *X. gladius* was observed with the Eco4-Mse2 primers combination. Here, all samples have a correlation with ladder higher than 0.99. For the males M6 and M7 , a peak at 272 bp is observed with an intensity between 500 and 1000 (Fig. 3). A peak seems also present at 272 bp for male M5 but it is not clear. On the other hand, for the 3 females this peak does not exist. A peak is detected by the software for the female F7 but it is certainly due to the high threshold (Fig. 4).

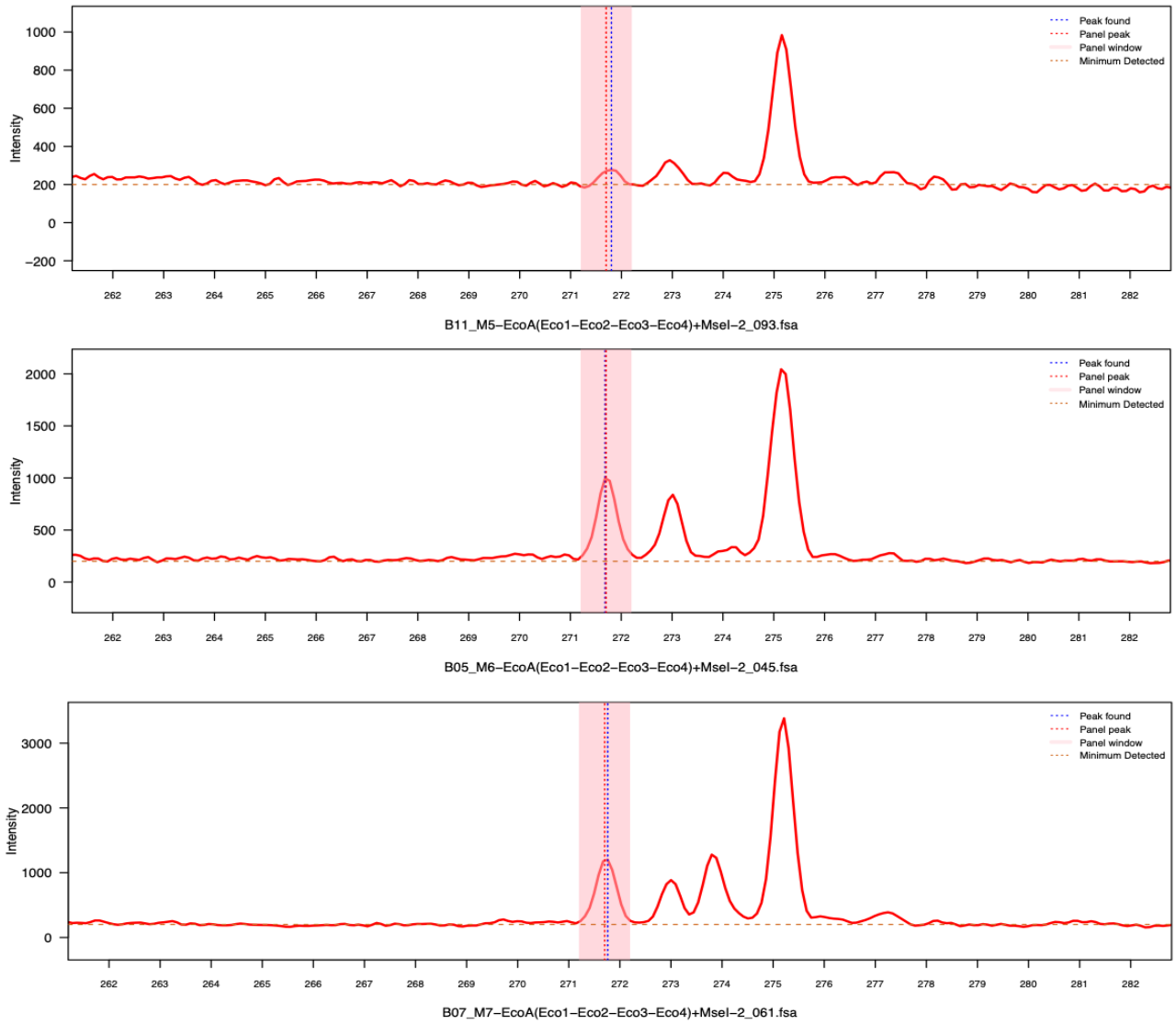


Figure 3 : Male DNA fragment intensities read at 272 bp for Eco4-Mse2

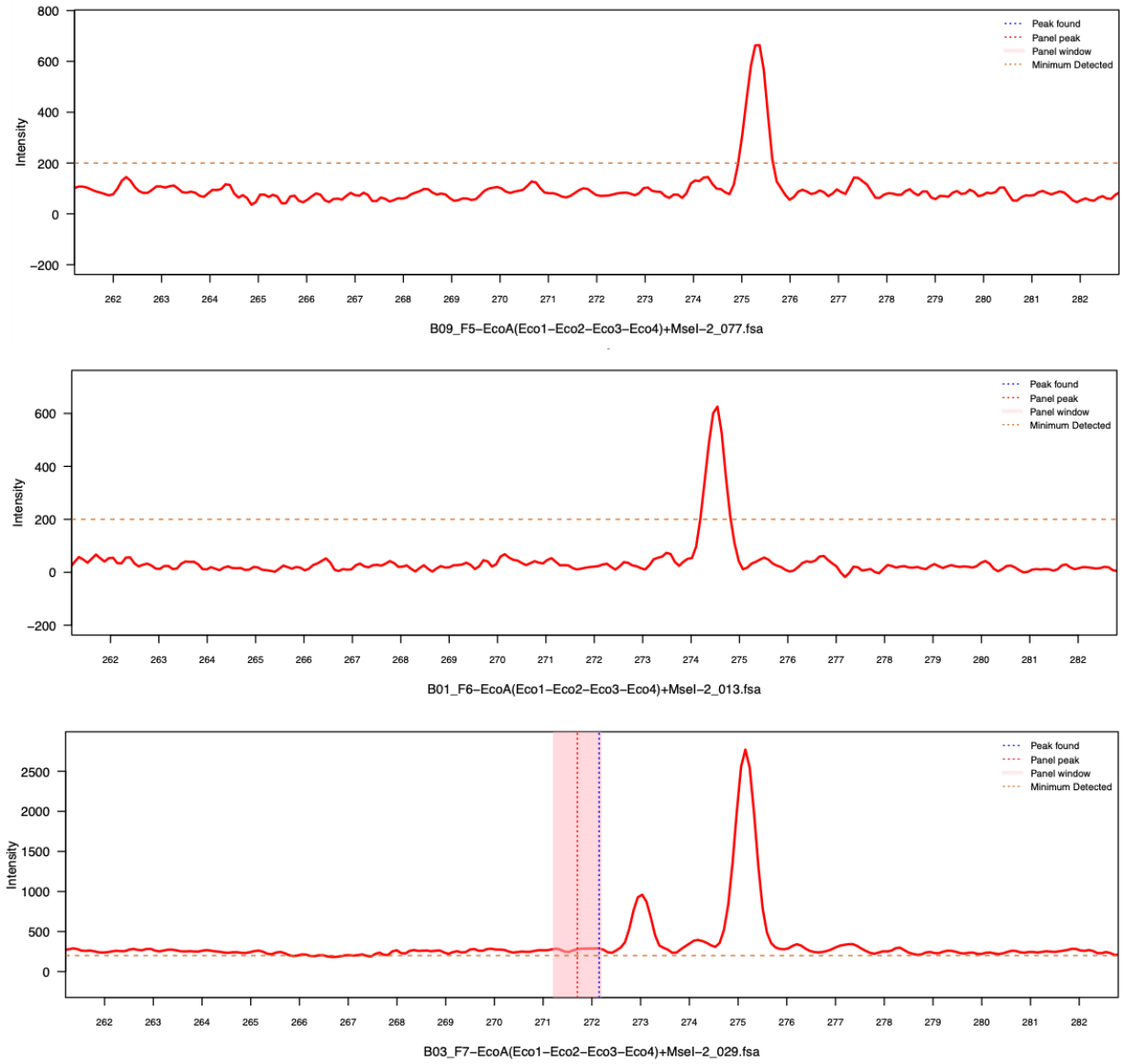


Figure 4 : Female DNA fragment intensities read at 272 bp for Eco4-Mse2

- **Combination Eco4 - Mse4:**

Another potential AFLP marker can be observed using the Eco4-Mse4 combination. In this case, sample F5 has a correlation of 0.9616 with the ladder whereas for M5 no correlation was found. Both of them were removed for the marker's determination. For the two other male (M6 & M7) a peak with high

intensity between 2000 and 2600 is observed at 165bp (Fig. 5). This peak was observed for only one female (F7) with a lower intensity around 536 which seems to be artefactual (Fig. 6).

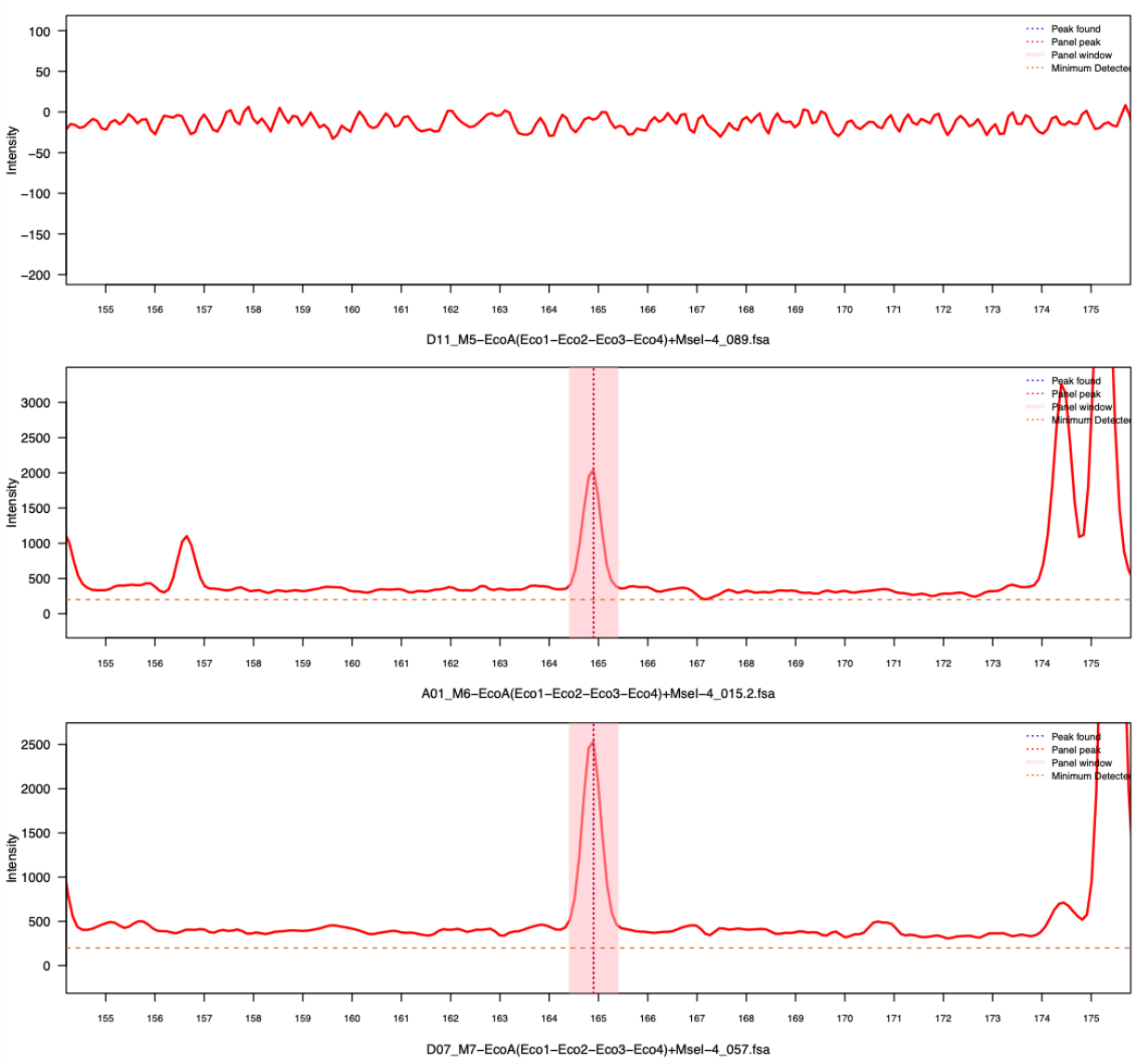


Figure 5 : Male DNA fragment intensities read at 165 bp for Eco4-Mse4

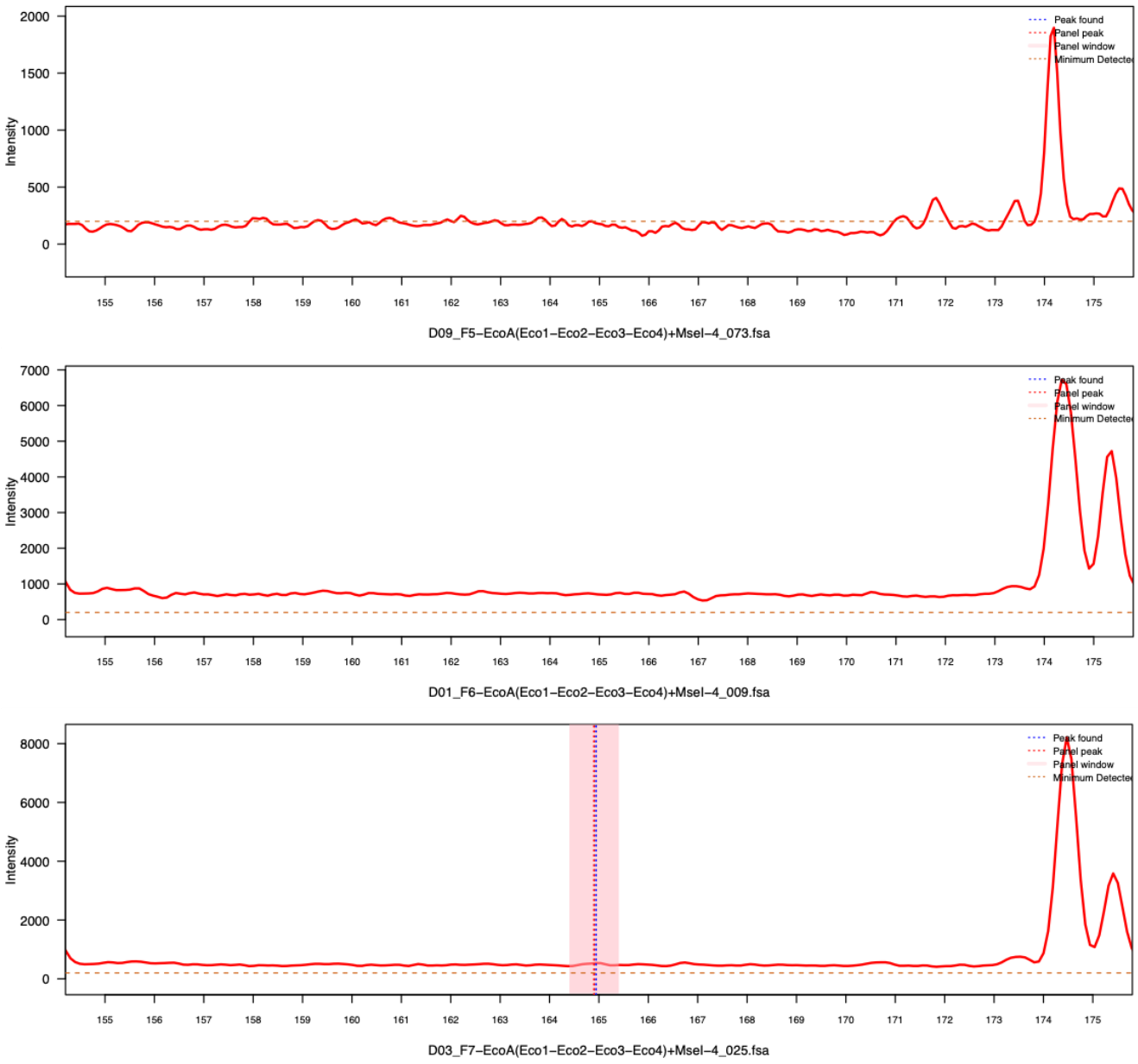


Figure 6: Female DNA fragment intensities read at 165 bp for Eco4-Mse4

Discussion

The preliminary analyses for sex determination of swordfish using a genetic method (AFLP) are very promising. We have identified 2 potential markers that could be used to easily identify the sex of swordfish individuals. This work is a first step to investigate the potential application of AFLP for sex identification of swordfish. We plan to extend the number of samples to 40 females and 40 males to confirm the results and identify robust markers of sex.

The samples from the Bengal Gulf showed some issues in quality as the correlation between the DNA fragment lengths and the reference ladder has a low correlation. This implies potential issues in DNA extraction. This issue should be solved by increasing the sample size of good DNA quality.

When AFLP will be done on more samples and sex specific loci will be clearly identified, AFLP fragments will be isolated and sequenced. As swordfish genome had been sequenced in February 2021 (scaffold assembly) (Wu et al., 2021) it would be possible to design specific primers in order to easily determine sex of swordfish by using only one PCR.

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 (~ 2-3h 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.

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Appendix

Table A1: Samples retained for AFLP

Name	Sex	Localisation
F5	Female	RUN
F6	Female	GBE
F7	Female	RUN
M5	Male	GBE
M6	Male	RUN
M7	Male	RUN

GBE : Gulf of Bengal, RUN : Réunion

Figure A2 : Electropherogram of the GeneScan™ - 600 LIZ Size Standard