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Use of Amplified Fragment Length Polymorphism and Sequence Characterized Amplified Region Marker for Identifying the Sex of the *Oxyeleotris marmorata*

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ABSTRACT

There is a huge demand for the *Oxyeleotris marmorata*, especially in Asian markets. However, farmers are unable to provide a constant supply of this fish to meet the demand, which is estimated to be around 100 metric tonnes per annum. One of the reasons that are hindering the supply is the low success rate of *O. marmorata* breeding programs. These breeding programs rely on many factors for their success, one of which is the use of genuine male and female adults, although determining these could be a daunting task. This research was carried out in an attempt to determine a sex marker for the *O. marmorata* using the amplified fragment length polymorphism (AFLP) method. Of the 30×30 AFLP primer mixtures screened, the E-TAA and M-CTT primer pair had an amplified ~600 bp marker that was specific to the female. This ~600 bp AFLP marker was later used to design a 464 bp sequence characterized amplified region (SCAR) marker. Thus, it has been suggested that the SCAR marker obtained has the potential to be used for the sexual identification of the *O. marmorata* at the juvenile stage, thereby enabling them to be used in breeding programs.

Keywords: AFLP, *Oxyeleotris marmorata*, sex determination, sex marker

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INTRODUCTION

In several Asian countries such as China, Thailand, Vietnam, and Malaysia, the *Oxyeleotris marmorata* (marble goby) is conventionally raised in cages located in freshwater waterways and ponds. It fetches between USD 20/kg to USD 50/kg in Asian markets (Herawati et al., 2016; Yong et al., 2013). Due to its pricey market value, the *O. marmorata* has the potential to be raised as an alternative profitable aquaculture species. Multiple efforts have been undertaken by various parties to perfect the culturing technique for the *O. marmorata* (Lam et al., 2014a, 2014b; Seetapan et al., 2012). However, the sexing of the *O. marmorata* is one of the biggest challenges faced by farmers in culturing this fish. False sexing of this fish will lead to failure in producing proper brood stocks for the breeding program. What is more challenging is that a system for determining the sex of this fish remains elusive.

The sexual dimorphism of the adult *O. marmorata* can be determined by observing the morphometric differences in its urogenital papilla, caudal fin and caudal peduncle. The female has a longer urogenital papilla in comparison to the male. On the other hand, both the caudal fin and caudal peduncle of the male are longer than those of the female (Idris et. al., 2012). The longer urogenital papilla observed in the female *O. marmorata* is attributed to the need to fulfil the spawning ritual, during which; the female uses its urogenital papilla to deposit its eggs on the surface of a substance. The longer caudal fin and caudal peduncle observed in the male *O. marmorata* are needed for adequate agitation and aeration for the mixing and milting of the eggs during the fertilization process (Idris et. al., 2012). This sexual dimorphism, however, is sometimes hard to determine by the untrained eye and can sometimes lead to false sexing in the field or farm.

In this study, the amplified fragment length polymorphism (AFLP) technique (Vos et. al., 1995) was proposed for the sex-specific marker identification of the *O. marmorata* using genomic DNA obtained from phenotypically sex-based morphometric features (Idris et al., 2012). The advantages of the AFLP are that it can be conducted without any prior sequence information; it has pre-designed generic primers, and a high multiplexing ratio. The AFLP had previously been successfully utilised for the sex identification of various organisms, including fishes such as the *Acipenser schrenckii*, *Clarias gariepinus*, *Scophthalmus maximus*, *Dicentrarchus labrax*, *Hypophthalmichthys nobilis*, *Hypophthalmichthys molitrix*, *Pseudobagrus ussuriensis*, *Pelteobagrus fulvidraco*, and *Oncorhynchus mykiss* (Cheng et al., 2013; Felip et al., 2000, 2005; Kovács et al., 2000; Liu et al., 2018; Pan et al., 2015; Vale et al., 2014; Xiao et al., 2014). To enable the sex marker to be used in breeding programs on a large scale in a more cost-efficient and labour-intensive manner, the AFLP marker was converted into a polymerase chain reaction-based marker such as the SCAR marker for marker-associated selections (Wang et al., 2011).

MATERIALS AND METHODS

Oxyeleotris marmorata Source and Genomic DNA Isolation

A total of 14 adult *O. marmorata* were collected from Empangan Kelau, Bentong Pahang, Malaysia (Coordinates 3° 34' 40.9224"N 101° 59'17.7504" E) using both fish traps and gill nets in the months of May 2018 and March 2019. The fish samples were kept alive at the Makmal Genomik 1, Universiti Kebangsaan Malaysia. The fish were kept in fish tank equipped with proper aeration and were fed once a day ad libitum with live feed. The determination of the sex of the *O. marmorata* was based on their morphometric features, as described by Idris et al. (2012). The lengths of the adult fish used in this study were between 20 to 30 cm, while their average weight was 300 g (± 30 g). The fish were separated into two groups. The first group, comprising 3 females and 3 males, was used for the construction of the gene pool, while the second group, comprising 4 males and 4 females, was used to verify the isolated sex markers. The care of the fish and all the experiments were conducted in compliance with the UKM Animal Ethics Guidelines, as approved by the UKM Ethics Committee (Animal Ethics approval number: FST/2018/MOHDSHAZRUL/28-MAR./905-MAR-2018-AUG-2019) on 11 April 2018. All the fish were euthanized at the end of the experiments using methods previously described by Blessing et al. (2010). The sexual organs were also observed to confirm the gender of the fish.

The genomic DNA was isolated from the caudal fin of the fish, according to the phenol-chloroform method with modifications (Sambrook et al., 1989). About 0.5 g of the caudal fin was taken from the *O. marmorata* and preserved in 95 % ethanol immediately after collection for storage prior to the genomic extraction process. The preserved sample was frozen using liquid nitrogen prior to homogenisation using a pre-chilled mortar and pestle. A total volume of 500 µL of extraction buffer (10 mM EDTA, 100 mM Tris-HCl, 200 mM NaCl, and 0.7% SDS) were added to the homogenised sample. The mixture was vortexed for 30 seconds. Proteinase K (50 µg) was added, and the mixture was incubated for 60 minutes at 50°C. Next, 650 µL of phenol, chloroform and isoamyl alcohol (25:24:1) were added to the sample, after which, the mixture was vortexed for 30 seconds, followed by centrifugation at 4°C for 5 minutes at a speed of 12, 000 x*g*. Later, the aqueous phase was transferred into a fresh tube, and the same volume of chloroform and isoamyl alcohol (24:1) was added to the sample. The mixture was vortexed for 30 seconds, followed by centrifugation at 4°C for 5 minutes at a speed of 12, 000 x*g*. The aqueous phase was transferred into a fresh tube, and two volumes of ethanol were added to the sample. The mixture was inverted several times and was incubated at -20°C for 16 to 20 hours. The mixture was then centrifuged at 4°C for 10 minutes at a speed of 12, 000 x*g*. The pellet was washed in 70% cold ethanol. The sample was then dried, and resuspended in sterile dH_2O containing

RNase A (10 µg/mL). The quality of all the genomic DNA obtained was checked using a NanoDrop™ Spectrophotometer ND-2000. Only the genomic DNA at a 260/280 ratio of 1.8 and above was used for all the assays.

AFLP Reaction Assay

Approximately 0.5 mg of genomic DNA from the first fish group was digested and ligated simultaneously at room temperature overnight using 0.5 M of NaCl, 5 U *Eco*RI (Promega Corporation), 10 U *Mse*I (New England Biolabs), 0.55 µL(1.0 mg/mL) of 100× BSA, 1 µL of *Mse*I adapter (20 pmol/µL), 1 µL of *Eco*RI adapter (20 pmol/µL), 200 U T4 DNA ligase (Promega), 1.1 µL of T4 DNA ligase buffer, and 5.5 µL of distilled water. The digested and ligated mixture was then diluted to 200 µL with Tris-EDTA buffer (20mM Tris-HCl, 0.1 mM EDTA) and kept at -20°C for use in the pre-amplification process.

For the pre-amplification process, a mixture was prepared with the following content: 7.5 µL of ligated DNA, 2.0 µL of 10× *Taq* polymerase buffer, 0.6 µL of 50 mM $MgCl_2$, 0.5 µL of 10 mM dNTPs (Invitrogen), 0.25 µL of *Eco*RI pre-amplification primer (20 pmol/µL), 0.25 µL of *Mse*I pre-amplification primer (20 pmol/µL), 2.5 U *Taq* polymerase (Invitrogen) and distilled water, to make a final volume of 20 µL. The cycling parameters used for the pre-amplification process were 94°C for 5 minutes, followed by 20 cycles at 94°C for 45 seconds, 56°C for 30 seconds, and 72°C for 2 minutes, followed by a final extension at 72°C for 30 minutes. The pre-amplification products were then diluted to 100 µL with Tris-EDTA buffer (20 mM Tris-HCl, 0.1 mM EDTA) and kept at -20°C for use in the selective amplification process.

Each 10 µL of the mixture for the selective amplification reaction contained 0.5 µL of the pre-amplified products, 1.0 µL of 10× *Taq* polymerase buffer, 0.6 µL of 50 mM $MgCl_2$, 0.5 µL of 10 mM dNTPs (Invitrogen), 1 µL of *Eco*RI + 3 primer (10 pmol/µL), 0.25 µL of *Mse*I + 3 primers (10 pmol/µL), 2.5 U of *Taq* polymerase (Invitrogen) and distilled water to make a final volume of 10 µL. The AFLP products were amplified using touchdown PCR. Stage 1: 94°C for 5 minutes (1 cycle); Stage 2: 94°C for 45 seconds, 65°C for 30 seconds, and 72°C for 2 minutes. The annealing temperature was then lowered by 1.0°C per cycle (10 cycles); Stage 3: 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 2 minutes (20 cycles); and this was followed by a final extension stage at 72°C for 15 minutes. The selective amplification products were separated in 1.5% agarose.

The sex-specific fragments were excised and purified from the agarose gel using NucleoSpin® Gel and PCR Clean-up Kit (MACHEREY-NAGEL), and were sent to Apical Sdn. Bhd. for sequencing.

Adaptation of AFLP Marker as SCAR Marker

Primer blast (NCBI) software was used in designing the SCAR primers for the AFLP marker sequences. These primers were used to amplify the genomic DNA of the second group of fishes, consisting of four males and

4 females. The SCAR reaction was carried out in 2.0 µL of 10× PCR reaction buffer, 0.5 µL of 25 mM $MgCl_2$, 0.6 µL of 2 mM dNTPs, 20 pmol each of forward and reverse primers, 0.5 µL of genomic DNA, 2.0 U *Taq* polymerase, 0.5 µL of genomic DNA and distilled water to bring it to a volume of 20 µL. The cycling parameters used were 94°C for 5 minutes, followed by 30 cycles at 94°C for 45 seconds, optimised annealing temperature for 45 seconds, 72°C for 75 seconds, and final extension cycle at 72°C for 15 minutes. All the PCR products were examined in 1.5% agarose to determine the success of the SCAR primer conversion.

RESULTS AND DISCUSSIONS

Combinations of 30×30 AFLP primer pairs (Appendix 1) were used to screen potential sex markers from groups of males and females of the *O. marmorata* genomic DNA. To verify that these markers were associated to the genomic sex DNA sequences, the genomic DNA from 6 male and female *O. marmorata* were used as the DNA templates for these primer pairs (Figure 1). The study showed that only the E-TAA and M-CTT primer pair specifically amplified the DNA amplicon size of ~600 bp from the female genomic DNA, making it a specific marker for the female *O. marmorata*. The ~600 bp amplicon was sequenced, giving 574 bp. The sequence was deposited at the GenBank with accession number: MW148239. However, from the nucleotide BLAST analysis that was done, the sequence revealed that the amplicon did not contain any homology to any other species sequence in the GenBank database (data not shown). The non-homology sequence was expected as the AFLP is able to detect random DNA variations and highly likely non-coding DNA (Figure 2).

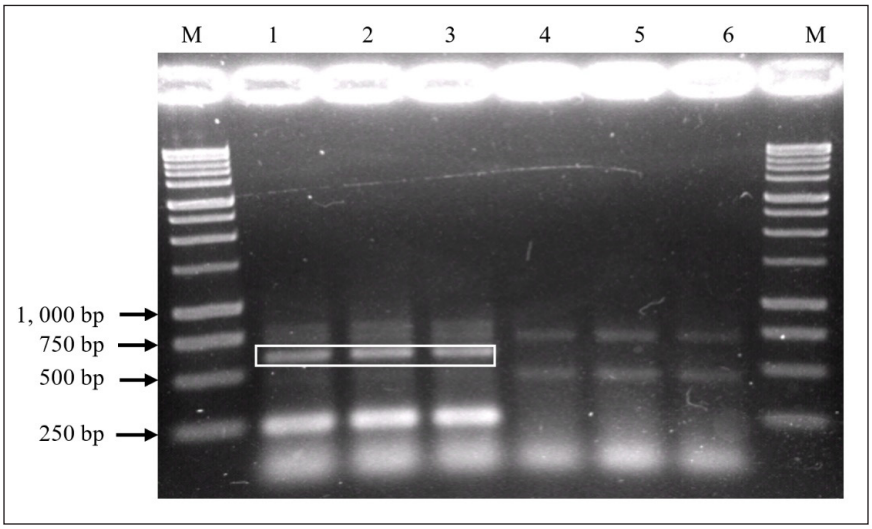

Figure 1. A ~600 bp band of the female-specific AFLP marker discovered with the E-TAA/M-CTT primer pair (marked in white box). Lanes 1 to 3 are the female samples; Lanes 4 to 6 are the male samples; Lane M is 1 kb DNA ladder marker

A primer pair (SM1: 5' GTCGGAATGTACCAAAGACAT 3' and SM2: 5'TATGAGTCCGTGAGTAACGC 3') was designed based on the sequence obtained from the female specific AFLP fragment from the E-TAA/M-CTT primer pair screening. The designed primer pair optimal annealing temperature was at 54°C, producing the expected ~480 bp amplicon, which was observed only in the female *O. marmorata*, but not in any male *O. marmorata* sample (Figure 3).

As far as is known, this is the first ever reported sex marker for the *Oxyeleotris marmorata* determined through the AFLP. The identified specific sex marker has multiple possible uses in basic research and commercial brood stock raising (Purcell et al., 2018). In basic research, the characterization of a sex marker for mature *O. marmorata* can provide invaluable data for understanding the sex determination mechanism. In commercial brood stock raising of the *O. marmorata*, the fish require

```
  1 GACTGCGTAC CAATTCTAAG AACCATAATA CATAAAGAAC AGGGCGAGCG GACAGCTCCC  60
 61 GGGCTCCATC CCTCCGGGGA GGCCCAACGT GTCGGAATGT ACCAAAGACA TCTATTATAC 120
                                    SM1
121 GGCCGAATCC TTAGATTTGA GCATCACTCC TCTCGCTGTG CGACACCCGG CGTATCTGAA 180
181 AGACACTTCG CACTTCATAG AGTCCATAAT GGATCTGCAC GTCCCGGGAG ACGCCTTCTT 240
241 CTTCTCCCTG GACATTGACT CTTTAAATAC CAACATTCCC ATCCAGGCCG GCATTCAGGC 300
301 CGTCAGAGAA ATTTTCCAAC TGTACCCTGA CCCCGCCAGG CCGGACGCGG AGCTCCTCCG 360
361 GTTACTGGAG ATCAATTTGA CTAAGAACGA CTTTGTCTTT GACGGCAAGT TCTACCTCCA 420
421 GGTCAAAGGG ACCGCCATGG GCAAACGCTT TGCCCCCGCT TACGCTAATA TCTTCATGGC 480
481 CAAATGGGAA AGACAAGTAC TGGCCACCTG CCCTAAAAAA CCGCTGCACT ACCTGCGTTA 540
541 CTCACGGACT CATACAAGTT ACTCAGGACT CATC
        SM2
```

Figure 2. The female-specific AFLP marker sequence and bold section indicate the primer designed for the SCAR marker

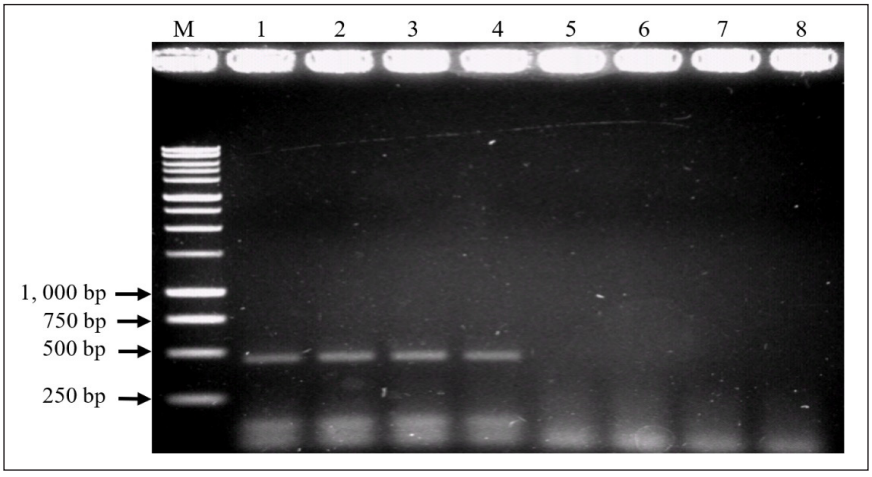

Figure 3. The SCAR marker for *Oxyeleotris marmorata* sexes determination. A ~ 480 bp band is present in the females (F) but not in the males (M). Lanes 1 to 4 are female sample. Lanes 5 to 8 are male sample. Lane M, 1 kb DNA ladder marker

almost 2 years before sexual identification can be done based on their reproductive organs, and only a highly-trained and experienced person will be able to do this as there are different distinct numbers of brood stocks to be maintained between the two sexes (Idris et al., 2012). Therefore, it will be more efficient and economical, in the long run if early sex identification can be done through molecular methods without having to wait for the fish to enter the maturity phase (Al-Ameri et al., 2016).

CONCLUSION

This study has succeeded in determining a female-specific AFLP and SCAR marker for *Oxyeleotris marmorata* samples from Tasik Klau, Bentong, Pahang. Both the female-specific AFLP and SCAR marker can be potentially used as a quick method for determining the sex of the *O. marmorata* for their potential use at the juvenile stage, thereby reducing the time and economic resources required to breed the species for commercialisation.

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APPENDIX 1

Supplementary Table
List of AFLP primer pairs used in the screening

No.	*Eco*RI AFLP primers (E-XXX)	No.	*Mse*I AFLP primers (M-XXX)
1	*5' GACTGCGTACCAATTCTAA 3'	1	5' GATGAGTCCTGAGTAAACA 3'
2	5' GACTGCGTACCAATTCTAT 3'	2	5' GATGAGTCCTGAGTAAACT 3'
3	5' GACTGCGTACCAATTCTAC 3'	3	5' GATGAGTCCTGAGTAAACC 3'
4	5' GACTGCGTACCAATTCTAG 3'	4	5' GATGAGTCCTGAGTAAACG 3'
5	5' GACTGCGTACCAATTCTTA 3'	5	5' GATGAGTCCTGAGTAAAGA 3'
6	5' GACTGCGTACCAATTCTTT 3'	6	5' GATGAGTCCTGAGTAAAGT 3'
7	5' GACTGCGTACCAATTCTTC 3'	7	5' GATGAGTCCTGAGTAAAGC 3'
8	5' GACTGCGTACCAATTCTTG 3'	8	5' GATGAGTCCTGAGTAAAGG 3'
9	5' GACTGCGTACCAATTCTCA 3'	9	5' GATGAGTCCTGAGTAACAA 3'
10	5' GACTGCGTACCAATTCTCT 3'	10	5' GATGAGTCCTGAGTAACAT 3'
11	5' GACTGCGTACCAATTCTCC 3'	11	5' GATGAGTCCTGAGTAACAC 3'
12	5' GACTGCGTACCAATTCTCG 3'	12	5' GATGAGTCCTGAGTAACAG 3'
13	5' GACTGCGTACCAATTCTGA 3'	13	5' GATGAGTCCTGAGTAACTA 3'
14	5' GACTGCGTACCAATTCTGT 3'	14	*5' GATGAGTCCTGAGTAACTT 3'
15	5' GACTGCGTACCAATTCTGC 3'	15	5' GATGAGTCCTGAGTAACTC 3'
16	5' GACTGCGTACCAATTCTGG 3'	16	5' GATGAGTCCTGAGTAACTG 3'
17	5' GACTGCGTACCAATTCAAA 3'	17	5' GATGAGTCCTGAGTAACCA 3'
18	5' GACTGCGTACCAATTCAAT 3'	18	5' GATGAGTCCTGAGTAACCT 3'
19	5' GACTGCGTACC AATTCAAC 3'	19	5' GATGAGTCCTGAGTAACCC 3'
20	5' GACTGCGTACCAATTCAAG 3'	20	5' GATGAGTCCTGAGTAACCG 3'
21	5' GACTGCGTACCAATTCATA 3'	21	5' GATGAGTCCTGAGTAACGA 3'
22	5' GACTGCGTACCAATTCATT 3'	22	5' GATGAGTCCTGAGTAACGT 3'
23	5' GACTGCGTACCAATTCATC 3'	23	5' GATGAGTCCTGAGTAACGC 3'
24	5' GACTGCGTACCAATTCATG 3'	24	5' GATGAGTCCTGAGTAACGG 3'
25	5' GACTGCGTACCAATTCACA 3'	25	5' GATGAGTCCTGAGTAAGAA 3'
26	5' GACTGCGTACCAATTCACT 3'	26	5' GATGAGTCCTGAGTAAGAT 3'
27	5' GACTGCGTACCAATTCACC 3'	27	5' GATGAGTCCTGAGTAAGAC 3'
28	5' GACTGCGTACCAATTCACG 3'	28	5' GATGAGTCCTGAGTAAGAG 3'
29	5' GACTGCGTACCAATTCAGA 3'	29	5' GATGAGTCCTGAGTAAGTA 3'
30	5' GACTGCGTACCAATTCAGT 3'	30	5' GATGAGTCCTGAGTAAGTT 3'

Note.
1. XXX represent the last 3 nucleotides of the primer
2. * The AFLP selective-primer pairs that were used for development of SCAR marker