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## Genotyping of red tilapia strains in Central Luzon Philippines targeting ESR1 gene

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### ABSTRACT

Tilapia is an economically important commodity worldwide. Marker-assisted selection (MAS) has been practiced in livestock culture to ensure the selection of a specific trait that will benefit the culture system. This study assessed the allelic variation in six red tilapia strains in Central Luzon, Philippines. Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) was done to evaluate the genotypes of six strains of red tilapia using intron one portion of the chromosome one of the estrogen receptor 1 (ESR1) gene. The target gene was amplified using primers designed in previous study and then subjected to RFLP using PvuII restriction enzyme. Single Nucleotide Polymorphisms (SNPs) were also evaluated in six DNA sequences. Results showed that 18 red tilapia samples were found to be of the AA genotype. Aligned sequences of the 122 bp ESR1 gene revealed that among six red tilapia strains, there are 117 identical pairs, three transversional pairs, and three transitional pairs. The SNPs observed can be used to identify restriction enzymes that discriminate different genotypes in the target gene. Association studies can be done to determine the genotypes that are linked to specific traits in cultured animals.

**Keywords:** Tilapia; PCR-RFLP; marker-assisted selection; PvuII; single nucleotide polymorphisms

### INTRODUCTION

Growing demands for food production and consumer demand for tilapia necessitate the search for alternate production methods for tilapia growth (Balcazar et al. 2004). Nile tilapia (*Oreochromis niloticus*) is perhaps Thailand's most essential cultured fish (Belton et al. 2006). Tilapia production accounted for 98,300 t, equivalent to 30% of recorded freshwater fish production (DOF 2005). In Malaysia, red tilapia

has dominated the total tilapia production. Ninety (90) percent of the total tilapia produced is accounted for red tilapia. National production increased by 26% between 2004 and 2007 (from 25,000 to 32,000 t) (Hamzah et al. 2009).

Among the genetically improved strains is the red tilapia. Red tilapia in the Philippines was introduced from Singapore in 1978 and subsequently crossed with *O. niloticus* from Taiwan, Japan, and Singapore (Galman et al. 1988). The original red



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tilapias were genetic mutants. A hybrid between a mutant reddish-orange female Mozambique tilapia and a regular male Nile tilapia produced the first red tilapia, known as Taiwanese red tilapia. In Florida, a red-gold Mozambique tilapia was generated by combining a normal-colored female Zanzibar tilapia with a red-gold Zanzibar tilapia. A hybrid between a mutant pink Nile tilapia and wild blue tilapia produced the third red tilapia strain discovered in Israel. All three original strains have been mated with unidentified red tilapia or wild *Oreochromis* species (Popma and Masser 1999).

Due to their fast growth, excellent salinity tolerance, appealing skin color, likeness to some marine species, and high price, red tilapias have acquired favor among aquaculturists (Watanabe et al. 1990). They are also suitable for both intensive and extensive settings, and their likeness to premium marine species has helped them gain consumer acceptability in various Asian countries (Gupta and Acosta 2004). Red tilapia could be a good choice for improvement because they can survive high salt and be raised in seawater (Vadhel et al. 2016). On the other hand, the development of diverse strains of red tilapia highlights the necessity to assess its genetic variability. Despite their widespread use and economic importance in tropical aquaculture, little is known about the genetic variation of red tilapia strains, particularly on other color variants (McAndrew et al. 1988).

According to Eknath et al. (1991), genetic improvement programs are beginning to be applied in aquatic species but far more left behind when compared to livestock and crops. For example, a genetic marker has been developed by Rothschild et al. (1991) that determines which sow will produce more offspring. A specific attribute required for successful breeding could be improved using molecular marker-assisted selection (Soller and Beckmann 1983). But before applying for marker-assisted selection programs, candidate genes or anonymous genetic markers associated with the target traits must be identified first.

Genotyping is the scientific method of differentiating an individual's genetic makeup (genotype) by examining its DNA arrangement. This method can identify minor genetic differences, which lead to significant variations in phenotype, comprising physical differences that make us unique and pathological changes underlying disease. This is traditionally used in breeding to find individuals with favorable alleles (Toyama et al. 2017). Estrogen receptor 1 (ESR1) is a gene known to control reproduction and development in most mammalian and non-mammalian vertebrates (Toyama et al. 2017).

This study assessed the intron of ESR1 with the use of PvuII restriction enzyme on six red tilapia strains in the Philippines. This method was developed by Rothschild et al. (1991) and was used to select pigs' (*Sus scrofa*) litter size. Evaluation of the same genotypic patterns was done for possible application of broodstock selection of different red tilapia strains. Single Nucleotide Polymorphisms (SNPs) were also identified for future use.

## METHODS

### Sample Collection

Muscle and fin tissues were taken from genetically modified farmed red tilapia in Central Luzon, Philippines. The red tilapia strains Muñoz and FAC Selected Tilapia (FaST) were obtained at the Freshwater Aquaculture Center (FAC) facilities. Batangas, Laguna, and Zambales strain were obtained from the College of Fisheries, Central Luzon State University (CF-CLSU). BFAR-Red strain was obtained from the Bureau of Freshwater and Aquaculture Center (BFAR). Both stations are located in the Science City of Muñoz, Nueva Ecija. Eighteen samples (triplicates in every strain) were used in this study (Table 1).

**Table 1.** Basic information of the red tilapia samples.

Strain	No. of Samples	Source	Genotype
Muñoz	3	FAC-CLSU	N/A
FaST	3	FAC-CLSU	N/A
Batangas	3	CF-CLSU	N/A
Laguna	3	CF-CLSU	N/A
Zambales	3	CF-CLSU	N/A
BFAR	3	BFAR	N/A

### DNA Extraction

Promega Wizard® Genomic DNA Purification Kit (USA) was used to extract fish genomic DNA according to the manufacturer's instructions. In a 15 ml centrifuge tube, 600 µl Nuclei

Lysis Solution was added and cooled on ice. Then, using a tiny homogenizer, about 20 mg of tissue samples were added to each tube with Nuclei Lysis Solution and homogenized for 10 s. In a 1.5 ml microcentrifuge tube, the lysate was transferred. The

tissue was pulverized in liquid nitrogen using a pre-chilled liquid nitrogen mortar and pestle. After the liquid nitrogen had evaporated, 10–20 mg of the powdered tissue was transferred to 600 µl of Nuclei Lysis Solution in a 1.5 ml microcentrifuge tube. The lysate was incubated for 15–30 min at 65°C.

The sample was mixed by inverting the tube 2–5 times after adding 3 µl of RNase Solution to the nuclear lysate. At 37 °C, the mixture was incubated for 15–30 min. Before starting, the sample was cooled down to room temperature for 5 min. Afterward, 200 µl of Protein Precipitation Solution was added to the sample and vortexed vigorously for 20 s at high speed. After cooling for 5 min on ice, the sample was centrifuged at 13,000–16,000 × g for 4 min. The protein pellet was transferred to a clean 1.5 ml microcentrifuge tube containing 600 µl of room temperature isopropanol after removing the supernatant containing the DNA. The solution was gently stirred by inversion until the white thread-like DNA strands formed a visible mass. This was centrifuged for 1 min at an ambient temperature at 13,000–16,000 × g. The supernatant was discarded, leaving the DNA as a pellet. Six hundred (600) µl of 70% ethanol was added, and the tube was inverted several times to wash and remove contaminants. This was centrifuged for 1 min at an ambient temperature at 13,000–16,000 × g. After placing the tube on clean absorbent paper, the pellet was air-dried for 10–15 min. The DNA was rehydrated with 100 µl of Tris-EDTA (TE) buffer and incubated at 65°C for 1 h. The DNA was stored at 2–8°C.

#### Polymerase Chain Reaction (PCR) Assay

The ESR1 gene was amplified using ESR1 F: 5'-CCT GTT TTT ACA GTG ACT TTT ACA GAG-3' and ESR1 R: 5'-CAC TTC GAG GGT CAG TCC AAT TAG-3' (Rothschild et al. 1991). The cocktail mix consisted of 3.0 µl 1X PCR buffer, 0.5 µl 2.5 mM MgCl<sub>2</sub>, 0.5 µl 2.0 mM dNTPs, 0.3 µl 10 pmol of each primer, 1.2 µl DNA sample, 1 unit of Taq DNA polymerase and 4.2 µl NFH<sub>2</sub>O.

Optimized PCR profile conditions for the ESR1 gene consist of 95°C for 5 min for the initial denaturation step, 35 cycles of: (95°C for 30 s) for denaturation, (62°C 30 s) for annealing, and (72°C 30 s) for extension and 72 °C for the final extension for 7 min. The PCR product was loaded to agarose gel for the result.

#### Restriction Fragment Length Polymorphism (RFLP)

Amplified ESR1 genes were digested using the PvuII restriction enzyme to identify the genotypes of each sample. A master mix composed of 1.7 µl nucleas-free H<sub>2</sub>O, 1.0 µl reaction buffer, 0.3 µl restriction enzyme, and 2.0 µl was used in the reaction. In a heat block, the mixture was incubated for 4 h at 37°C. Gel electrophoresis was used to confirm the result.

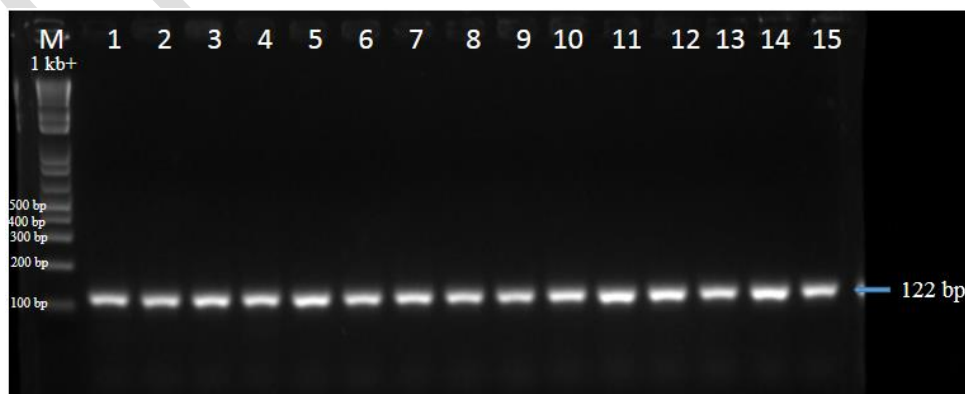
#### Gene Sequencing and Phylogenetic Analysis

Six DNA samples were sent to 1st Base (Malaysia) for sequencing with one for each red tilapia strain. MEGA 7.1 software was used to align and compare sequences. MN586879–MN586884 are the accession numbers for the nucleotide sequences submitted to GenBank. Single Nucleotide Polymorphisms (SNPs) in the six samples were observed. As to phylogenetic analysis, there were six sequences aligned using the CLUSTAL W alignment tool under Molecular Evolutionary Genetics Analysis (MEGA) 7 software. Then, the evolutionary history was inferred using Neighbor-Joining method, whereas the evolutionary distances were computed using the Jukes-Cantor model with 1,000 bootstrap replications.

## RESULTS

#### PCR

The ESR1 gene was amplified from all the samples with an amplicon size of 122 base pairs, as shown in Figure 1. The PCR products were used for the RFLP analysis using the PvuII restriction enzyme.

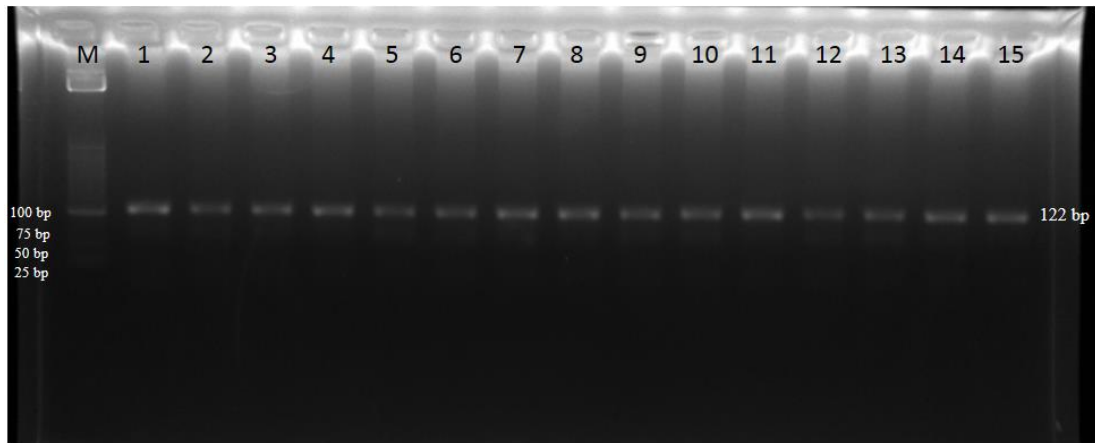


**Figure 1.** Agarose gel image of amplified product of ESR1 gene from red tilapia.

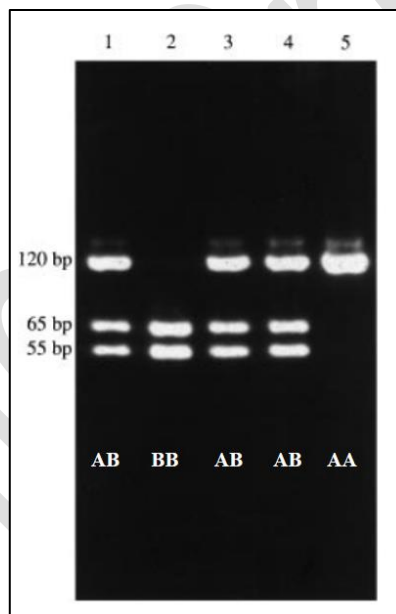
### Restriction Fragment Length Polymorphism (RFLP)

Restriction Fragment Length Polymorphism (RFLP) analysis of the ESR1 gene using PvuII showed

only one band pattern among all samples (Figure 2). Results showed that all samples are of the AA genotype (one band at 122 bp). Figure 3 shows the single band pattern that can be observed.



**Figure 2.** Agarose gel image of PvuII digested PCR product of ESR1 gene from red tilapia.



**Figure 3.** Three types of alleles observed in intronic ESR1 region of *Sus scrofa* cut using PvuII (Short et al. 1997).

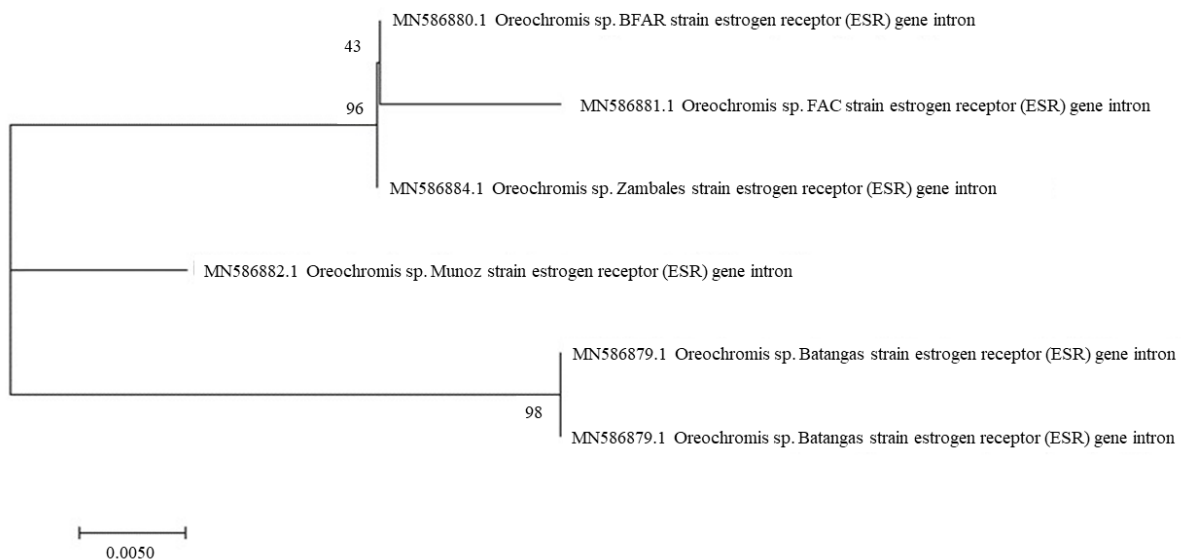
### Sequence and Phylogenetic Analysis of ESR1

A total of six sequences (one representative per strain) of ESR1 from red tilapia were generated. The amplified ESR1 gene produced a 122 bp sequence (Figure 3). Comparing all six sequences, there were 117 identical pairs, three transitional pairs, and three transversional pairs with a ratio of 1.9 (Figure 4). Phylogenetic analysis (Figure 5) did not consist of an outgroup since no ESR1 gene from other fish were

deposited in National Center for Biotechnology Information. Meanwhile, the same fragment isolated from pigs were deposited in NCBI. However, the genetic distance from both species could be questionable. The phylogenetic tree showed 3 clades between the 6 red tilapia strains putting Laguna and Batangas strain together while BFAR, FAC and Zambales in different clade and Muñoz strain was isolated from the 2 groups.

#MN586879_O_niloticus_ESR1_gene_Batangas_strain	CCTGTTTITA CAGTGACTTT TACAGAGTAT ATCTAAGAT GCAGAATCAA GTTTTATGAG ACCAACTTTC TTGTCAAGTC	[ 80]
#MN586880_O_niloticus_ESR1_gene_BFAR_strain	.....G-T C.....-	[ 80]
#MN586881_O_niloticus_ESR1_gene_FaST	.....G-T C.....-	[ 80]
#MN586882_O_niloticus_ESR1_gene_Munoz	.....-T C.....	[ 80]
#MN586883_O_niloticus_ESR1_gene_Laguna	.....- C.....	[ 80]
#MN586884_O_niloticus_ESR1_gene_intron_Zambales	.....G-T C.....-	[ 80]
#MN586879_O_niloticus_ESR1_gene_Batangas_strain	CCC-C-TATT CCACCTTATT CTAATTGGAC TGACCTCTGA AGTG	[124]
#MN586880_O_niloticus_ESR1_gene_BFAR_strain	...ATGC...	[124]
#MN586881_O_niloticus_ESR1_gene_FaST	.A.ATG...	[124]
#MN586882_O_niloticus_ESR1_gene_Munoz	...T.--...	[124]
#MN586883_O_niloticus_ESR1_gene_Laguna	...-.A....	[124]
#MN586884_O_niloticus_ESR1_gene_intron_Zambales	...ATGC...	[124]

**Figure 4.** Aligned sequences of six ESR1 gene from red tilapia strains in the Philippines.



**Figure 5.** Phylogenetic analysis of the 6 red tilapia strains in the Philippines.

## DISCUSSION

Estrogens are crucial for females and males in terms of fertility and reproductive capacity in vertebrates, and their signal is controlled by ESRs (Yan et al. 2019). This study determined the allelic variation in the ESR1 gene of 6 red tilapia strains.

The ESR1 gene was successfully amplified in the 18 individual red tilapia samples. However, only one band pattern was observed during RFLP analysis: the AA genotype (single band at 122 bp). It can be justified by the sequences, which show that the cutting site (5'-CAGCTG-3') for PvuII is not present in the same region in the red tilapia ESR1 gene. Studies on pigs showed that genotype AA is the most common genotype. The genotype BB (favorable genotype) has low rate of heritability in pigs, which can be true in the case of Tilapia, hence the result of this study revealed genotype AA in 18 samples. One of the limitations is the availability of related studies using ESR1 gene as marker in Tilapia which only indicates that this study serves as a baseline study. Moreover, repository sequences found in NCBI are limited to ESR1

reference sequences in pigs and sequences obtained from the result of this study in Tilapia. In the study conducted by Yan et al. (2019), they found out that the ESR1 gene in Nile tilapia has no phenotypes of reproductive development and function in both females and males. It can be the reason why genotypes AB (banding patterns at 120, 65, and 55 bp) and BB (banding patterns at 55 and 65 bp) (Figure 3) were not observed in the result of the RFLP analysis. In quail, the study showed that the polymorphism in the ESR1 gene related to egg numbers was found in exon 8 (Wu et al. 2015). While in Chinese dagu chicken, C/T transition located within exon 4 of the ESR1 gene was found to have an association with egg production. The presence of this allelic variation differs in each species. Association studies must be conducted to locate these SNPs associated with specific traits (i.e., high fecundity in tilapia).

Though the cutting site for PvuII was not present in the sequences, the presence of some polymorphisms was observed in the amplified ESR1 intronic region. Out of 122 nucleotides, only 116 bp



have identical pairs among the samples. Transversional pairs were observed in loci numbers 68, 83, and 86, while transitional pairs occurred in loci 71, 72, and 88. Insertion-deletion (Indels) were observed in loci number 69, 70, 79 and 85-88. These polymorphisms can somehow be the subject of future studies in finding other suitable restriction enzyme/s for the genotyping of this gene. Furthermore, data on polymorphism in this gene can be used in association studies.

Additionally, the sequences generated in this study were analyzed for SNPs. These SNPs can help find another restriction enzyme/s that can show different alleles in the six different strains of red tilapia. Phylogenetic analysis revealed 3 different clades from the 6 strains of red tilapia. However, these genetic variations did not contribute to their genotypic characteristics.

The observed genotypes can be associated with favorable traits, such as high fecundity and a high fry survival rate. However, this requires extensive analysis. According to Vicencio et al. (2017), litter size is one of the factors which is essential in measuring reproductive success of a sow in swine operations. On the other hand, fecundity is the measure of reproductive success in Tilapia. Hence this study was conducted to evaluate ESR1 gene as candidate marker of fecundity in Tilapia. Therefore, it can be attributed as the most important economic trait in aquaculture production and brings more economic profit for the fisheries industry. The development of such technology will directly benefit the aquaculture industry as it will be easier for the farmers to look for the best broodstocks to be used in breeding in the future.

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## ETHICAL CONSIDERATIONS

This study followed all institutional and national ethical guidelines for the care and use of tilapia for the sample collection.

## DECLARATION OF COMPETING INTEREST

The authors declare that there are no competing interests to any authors.

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**ROLE OF AUTHORS:** ACMDG – conduct of the experiments; KJC –manuscript editing; kdda – sampling; mrudg –manuscript writing and editing.