Veterinary Integrative Sciences 2022; 20(1): 73 - 83. DOI; 10.12982/VIS.2022.007

Vet Integr Sci

Veterinary Integrative Sciences

ISSN; 2629-9968 (online)

Website; www.vet.cmu.ac.th/cmvj

Research article

# Association of genetic markers with sex determination in Thai red tilapia

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## Abstract

The objectives of this study were to verify the polymorphism on sex-linked marker loci and to assess their associations with phenotypic sex characteristics in red tilapia. Four sex-linked genetic markers of *Amh*, *SCAR4*, *SCAR5*, and *Oni3161* were genotyped in the Thai red tilapia population. The *Amh* marker was significantly associated with the phenotypic sex of red tilapia with an accuracy of 46.2%. No significant association of *SCAR4*, *SCAR5*, and *Oni3161* marker polymorphisms with phenotypic sex characteristics was observed in this study. However, the combinations of these two, three, and four markers were increasingly associated with phenotypic sex characteristics for red tilapia with an accuracy of 62.8, 68.4, and 72.4%, respectively. These results indicate that these combined genetic markers were associated with the phenotypic sex of red tilapias. These findings confirmed the importance of these genetic markers as candidate markers for sex determination in the Thai red tilapia population.

**Keywords:** Genetic markers, Red tilapia, Sex determination

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**Funding**; This research project is supported by Chiang Mai University and is partially supported by the Center of Excellence on Agricultural Biotechnology, Office of the Permanent Secretary, Ministry of Higher Education, Science, Research and Innovation (AG-BIO/MHESI).

***Article history;*** *received manuscript: 23 September 2021,*
*revised manuscript: 6 October 2021,*
*accepted manuscript: 25 October 2021,*
*published online: 26 October 2021*
***Academic editor;*** *Korakot Nganvongpanit*

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# INTRODUCTION

Nile tilapia (*Oreochromis niloticus*) is one of the most cultivated and economically important species in aquaculture in the world (Cáceres et al., 2019). Male monosex tilapia cultures are preferred to female because of the differential in growth rate and better feed conversion ratio (El-Greisy and El-Gamal, 2012). Currently, the widely used method to obtain all-male populations is the use of hormones in feeding during larval and fry phases for sex reversal (Rosenstein and Hulata, 1994; Mair et al., 1997). However, these methods are relatively expensive, need high levels of control, leave chemical residues, and affect public perceptions (Beardmore et al., 2001). An alternative approach has demonstrated that a breeding program incorporating hormonal-induced sex reversal has successfully generated the progeny YY male genotypes, known as genetically male tilapia (Mair et al., 1997; Sun et al., 2014). Thus, identifying genomic regions associated with sex determination in Nile tilapia is a research topic of great interest (Cáceres et al., 2019). The various genomic approaches of quantitative trait loci (QTL) and physical mapping have been carried out to identify QTL for sex determination in Nile tilapia populations (Eshel et al., 2012; Palaiokostas et al., 2013; Sun et al., 2014). Currently, the genome-wide association study (GWAS) and whole-genome sequencing (WGS) have been used to identify candidate genes for sex determination in Nile tilapia (Cáceres et al., 2019; Triay et al., 2020). Numerous strong candidate genes for involvement in sex determination of Nile tilapia have been described e.g., anti-Müllerian hormone (*Amh*), doublesex- and mab-3 related transcription factor 2 (*Dmrt2*), Wilms tumor suppressor protein 1b (*WT1b*), and cytochrome P450 family 19 subfamilies A member 1 (*CYP19A1*) genes (Shirak et al., 2006; Lee and Kocher, 2007). Moreover, several sex-linked genomic regions have been localized in Nile tilapia, including associated regions on linkage groups (LG) 1, 3, 20, and 23 (Shirak et al., 2006; Eshel et al., 2012; Palaiokostas et al., 2013; 2015; Conte et al., 2017). However, information on the association of the genetic markers with the phenotypic sex characteristics of Thai red tilapia has been limited. In the present study, we have verified the polymorphic sites of the genetic markers on LG20 and LG23, while their association with phenotypic sex characteristics has also been assessed in the Thai red tilapia population.

# MATERIALS AND METHODS

## Fish and DNA isolation

A total of 150 red tilapias (*Oreochromis niloticus* × *Oreochromis mossambicus*) (75 males and 75 females) were obtained from the Uttaradit Aquaculture Genetics Research and Development Center, Department of Fisheries, Thailand. The fish were fed on pelleted commercial diet containing 30% crude protein and 3% fat content with 3 to 5% levels of their body weight. At 5 months of age, the fish were harvested according to standard commercial procedures. According to the Animals for Scientific Purposes Act, B.E. 2558 (2015), since a part of this experiment was performed on carcass of animals, no ethical approval was required for this study and confirmed by the Animal Ethics Committee. Fin tissues of all fish were collected from their carcass and

phenotypic sex characteristics were observed. DNA samples were extracted from fin tissues using the standard phenol-chloroform method and were kept at 4 °C until analysis. The degree of concentration of the DNA sample was measured with a Nanodrop 2000c spectrophotometer (Thermo Scientific, USA) and the integrity of the DNA was checked on 1% agarose gel electrophoresis.

## Genotyping of genetic markers

Four sex-linked genetic markers of Nile tilapia were selected from previous studies consisting of anti-Müllerian hormone (*Amh*) on LG23 (Eshel et al., 2014), sequence characterized amplified region 4 (*SCAR4*) and *SCAR5* on LG23 (Sun et al., 2014), and *Oni3161* on LG20 (Palaiokostas et al., 2015). These markers were used to verify the polymorphism in the Thai red tilapia population. The specific primers of the *Amh* gene were designed based on relevant nucleotide sequence information (GenBank accession number: HG518787.1 and HG518784.1). The specific primers of *SCAR4* and *SCAR5* were designed based on nucleotide sequence information of the GenBank (KC710221.1, KC710222.1, KC710223.1, and KC710224.1), as well as the specific primers of *Oni3161* were designed based on the nucleotide sequence in the Ensembl database (NC_031984.2: g.16097335-16097494) as is shown in Table 1. A mismatched primer was designed to introduce a recognition site of the restriction enzyme for genotyping (Table 1). These four markers were genotyped using the polymerase chain reaction (PCR) amplification or PCR-fragment length polymorphism (PCR-RFLP) assay. The PCR was carried out in a total volume of 20 µl consisting of 50 ng of genomic DNA sample, $1\times(NH_4)_2SO_4$ buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 mM each primer (Table 1), and 0.2 U *Taq* DNA polymerase (Fermentas, USA). The PCR conditions were as follows: at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 58-62 °C for 30 sec, elongation at 72 °C for 45 sec, and then 5 min at 72 °C to complete the reaction. The PCR products were digested with a restriction enzyme (Table 1). The PCR products and digested products were electrophoresed on 6% polyacrylamide gels and stained with ethidium bromide for visualization (Norseeda et al., 2021).

**Table 1** Primer sequences, PCR condition, and restriction enzyme used for genotyping of the sex-linked markers.

| Primer | Nucleotide sequence | Tm (°C) | Product size (bp) | Restriction enzyme |
|---|---|---|---|---|
| *Amh* | F: 5′-TCTGCTGAAGGCTCTCTGCAG-3′ | 62 | 153/386 | - |
| | R: 5′-AGCTCTAGCGGCATCCACA-3′ | | | |
| *SCAR4* | F: 5′-AAACAACTGCATGGCCCTAC-3′ | 60 | 117/477 | - |
| | R: 5′-AAGATGGGAAGGCTTTCCAC-3′ | | | |
| *SCAR5* | F: 5′-CTGCAGTCCTTATAAACCAG-3′ | 60 | 165/201 | - |
| | R: 5′-GCAATGAATTCAAAGCCAGTG-3′ | | | |
| *Oni3161** | F: 5′-CTCTTTGAGTTATTTTAGTTGG-3′ | 58 | 160 | *Hae*III |
| | R: 5′-CTTTAACCCTCTCACTTAACT-3′ | | | |

*Mismatched nucleotide is underlined to generate a recognition site of the restriction enzyme *Hae*III for genotyping.

### Statistical analysis

The genotype and allele frequencies of the genetic markers were calculated. Associations of the genetic markers with phenotypic sex of red tilapia were estimated by binary logistic regression model as follows: $Y_{ijkl} = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + e_{ijkl}$ and Y = ln (P/1-P) where P is the probability of classification as male. Note that the denominator (1-P) is the probability of classification as female. If the predicted value is equal to or more than 0.5, it is considered males and if it is less than 0.5, it is considered to be females; $\beta_0$ = the intercept; $\beta_1$, $\beta_2$, $\beta_3$, $\beta_4$ = the regression coefficients for the genotype of genetic markers 1, 2, 3, and 4; $X_1$, $X_2$, $X_3$, $X_4$ = the dummy variables for the presentation of effects of the genotype of markers 1, 2, 3, and 4; and $e_{ijkl}$ = random error term. Moreover, an accuracy test was estimated as ascribed by a previous report (Wassertheil-Smoller, 2004).

## RESULTS

### Polymorphisms of genetic markers

Four genetic markers (*Amh*, *SCAR4*, *SCAR5*, and *Oni3161*) were found to be segregating among this Thai red tilapia population. The *Amh*, *SCAR4,* and *SCAR5* markers were insertion (Ins) and deletion (Del) polymorphisms and were detected with PCR and gel electrophoresis. The *Oni3161* marker was a single nucleotide polymorphism (SNP) and was detected with the restriction enzyme *Hae*III. The *Amh* marker showed two motif alleles presented a 386-bp fragment for as allele A (Ins) and a 153-bp for allele B (Del) as shown in Figure 1. The *SCAR4* marker showed two specific alleles revealed a 477-bp fragment for as allele A (Ins) and a 117-bp for allele B (Del) (Figure 2). The *SCAR5* marker showed two specific alleles revealed a 201-bp fragment for as allele A (Ins) and a 165-bp for allele B (Del) (Figure 3). The *Oni3161* marker showed two specific alleles exhibited two fragments of 138 and 22-bp for allele A (T) and a 160-bp fragment for allele B (C) (Figure 4).

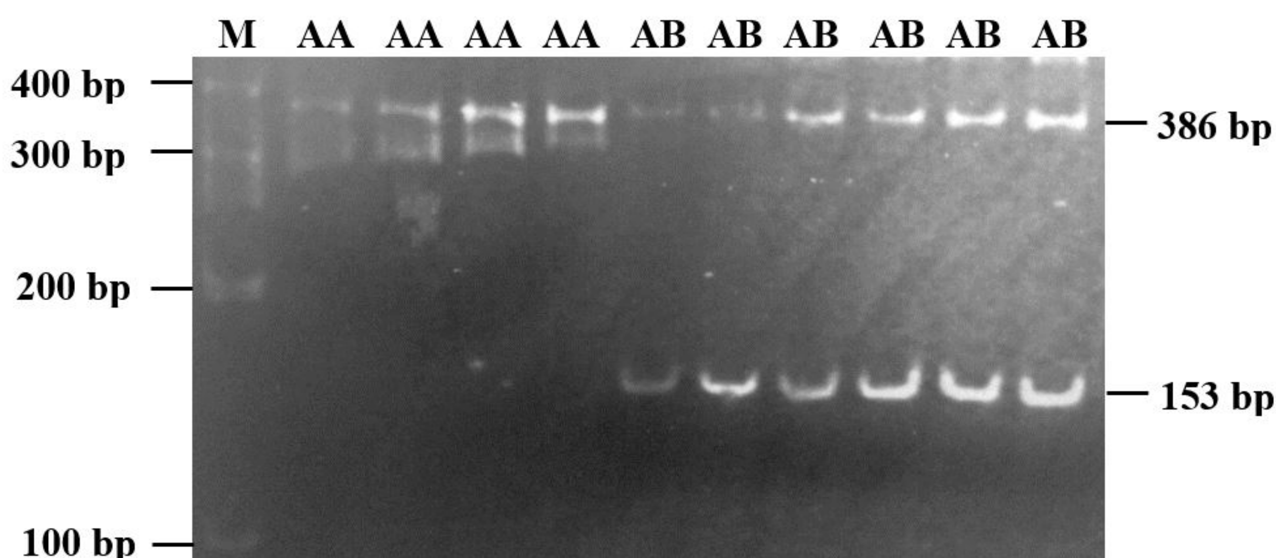


**Figure 1** Genotyping of the *Amh* marker. The molecular marker of the 100 bp DNA ladder (M) and the genotypes of the *Amh* marker are indicated at the top of each line. A 386-bp fragment for allele A (insertion, Ins) and a 153-bp fragment for allele B (deletion, Del).

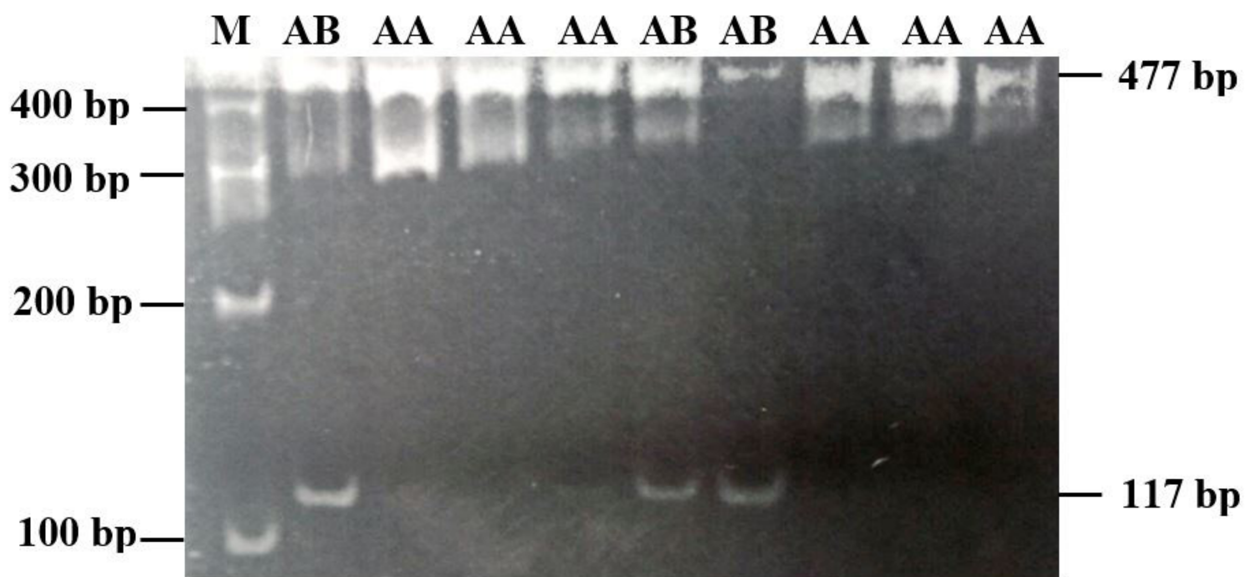


**Figure 2** Genotyping of the *SCAR4* marker. The molecular marker of the 100 bp DNA ladder (M) and the genotypes of the *SCAR4* marker are indicated at the top of each line. A 477-bp fragment for allele A (insertion, Ins) and a 117-bp fragment for allele B (deletion, Del).

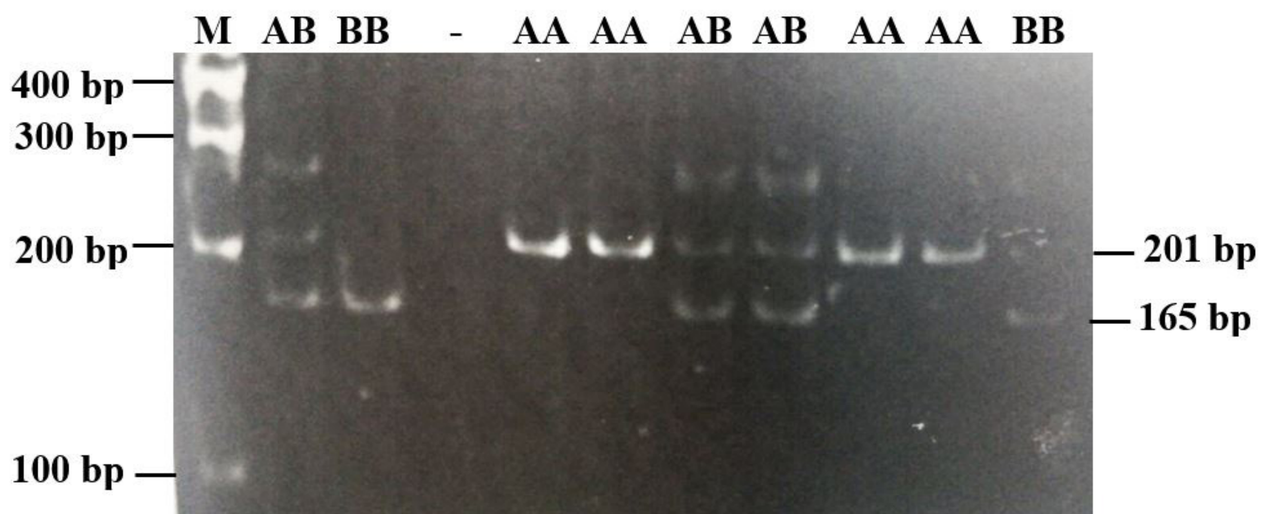


**Figure 3** Genotyping of the *SCAR5* marker. The molecular marker of the 100 bp DNA ladder (M) and the genotypes of the *SCAR5* marker are indicated at the top of each line. A 201-bp fragment for allele A (insertion, Ins) and a 165-bp fragment for allele B (deletion, Del).

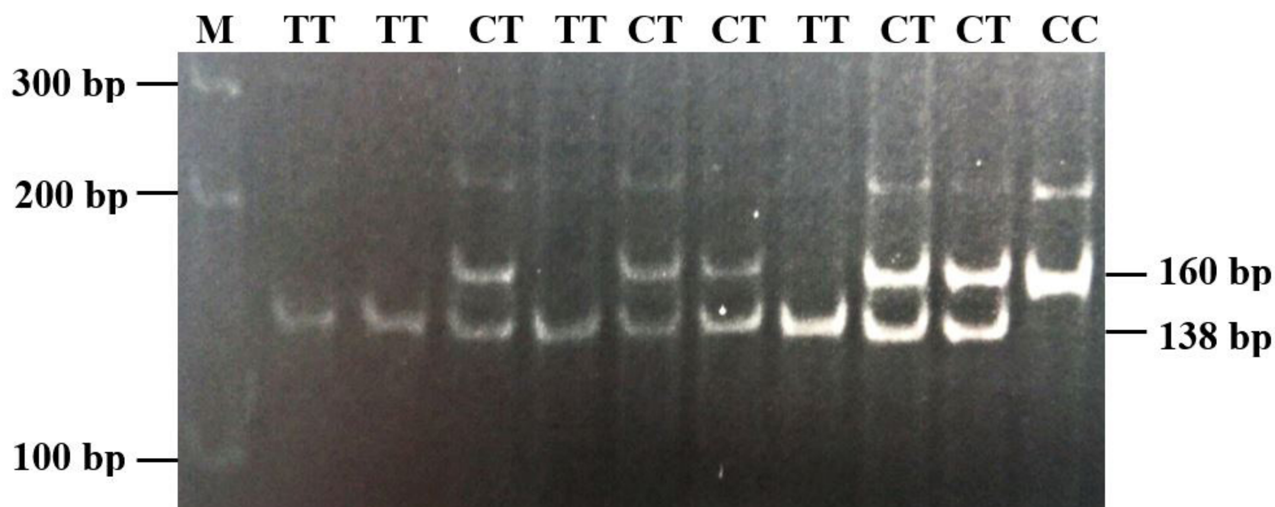


**Figure 4** Genotyping of the *Oni3161* marker. The molecular marker of the 100 bp DNA ladder (M) and the genotypes of the *Oni3161* marker are indicated at the top of each line. A 160-bp fragment for allele C and two fragments of 138 and 22-bp for allele T. Notably, the 22-bp fragment is not shown in the gel.

## Genotype and allele frequencies

The genotype frequencies of the genetic markers for the red tilapia are shown in Table 2. At the *Amh*, *SCAR4*, and *SCAR5* loci, two genotype patterns were presented as Ins/Ins (AA) and Ins/Del (AB). The frequencies of AA and AB genotypes of the *Amh* marker were 0.54 and 0.46 in this red tilapia population. The AA genotype of the *SCAR4* locus had higher frequencies than the AB genotype. On the other hand, the AB genotype of the *SCAR5* locus had higher frequencies than the AA genotype. At the *Oni3161* locus, three genotypes were segregating in the Thai red tilapia population. The AA and AB genotypes had higher frequencies than the BB genotype. Moreover, the allele frequencies of these four marker loci showed allele A had higher frequencies than allele B.

**Table 2** Genotype and allele frequencies for sex-linked genetic markers in Thai red tilapia.

| Markers | Genotype frequencies | | | Allele frequencies[1] | |
|---|---|---|---|---|---|
| | AA | AB | BB | A | B |
| *Amh* | 0.54 | 0.46 | - | 0.77 | 0.23 |
| *SCAR4* | 0.73 | 0.27 | - | 0.86 | 0.14 |
| *SCAR5* | 0.39 | 0.61 | - | 0.70 | 0.30 |
| *Oni3161* | 0.50 | 0.40 | 0.10 | 0.70 | 0.30 |

[1]Allele A represents wild-type alleles of *Amh*Ins, *SCAR4*Ins, *SCAR5*Ins, and *Oni3161*T for each locus and allele B represents mutate alleles of *Amh*Del, *SCAR4*Del, *SCAR5*Del, and *Oni3161*C for each locus.

## Association of genetic markers with phenotypic sex characteristics of Thai red tilapia

The association of genetic markers (*Amh*, *SCAR4*, *SCAR5*, and *Oni3161*) with phenotypic sex characteristics of the Thai red tilapia population is shown in Table 3. The *Amh* marker was significantly associated with phenotypic sex characteristics of red tilapia ($P$ = 0.0177) with an accuracy of 46.2%. The *SCAR4* marker showed a trend toward being associated with phenotypic sex characteristics with an accuracy of 31.4% ($P$ = 0.1522). While the *SCAR5* and *Oni3161* markers showed no association with phenotypic sex characteristics of red tilapia. Moreover, the combinations of these two, three, and four markers were increasingly associated with phenotypic sex characteristics of the red tilapia with a highest accuracy of 62.8, 68.4, and 72.4%, respectively (Table 3). However, the other combinations of two and three markers were also associated with phenotypic sex characteristics of the red tilapia with an accuracy of 44.4 to 58.5% and 56.0 to 65.4%, respectively.

**Table 3** Equations and association of genetic markers with phenotypic sex characteristics and the sex prediction accuracy rate in Thai red tilapia.

| Markers (X) | Equation[1] | Likelihood Ratio Test | *P*-value | Accuracy (%)[2] |
|---|---|---|---|---|
| *Amh* ($X_1$) | $y = 1.0986 - 1.5041\ X_1$ | 5.62 | 0.0177 | 46.2 |
| *SCAR4* ($X_2$) | $y = 0.6466 - 0.9831\ X_2$ | 2.05 | 0.1522 | 31.4 |
| *SCAR5* ($X_3$) | $y = 0.4155 - 0.0805\ X_3$ | 0.02 | 0.8641 | 29.7 |
| *Oni3161* ($X_4$) | $y = 0.1178 + 0.4128\ X_4$ | 0.43 | 0.5111 | 29.1 |
| $X_1 + X_2$ | $y = 1.5404 - 1.6739\ X_1 - 1.2527\ X_2$ | 8.39 | 0.0150 | 62.8 |
| $X_1 + X_2 + X_3$ | $y = 1.5510 + 1.6736\ X_1 - 1.2507\ X_2 - 0.0189\ X_3$ | 9.00 | 0.0293 | 68.4 |
| $X_1 + X_2 + X_3 + X_4$ | $y = 1.0314 - 1.6893\ X_1 - 1.3337\ X_2 + 0.2259\ X_3 + 0.6814\ X_4$ | 9.14 | 0.0476 | 72.4 |

[1]y is the probability of the prediction of the Thai red tilapia by the genetic markers. $X_1$, $X_2$, $X_3$, and $X_4$ refer to AA, AB, and BB genotypes of the *Amh*, *SCAR4*, *SCAR5*, and *Oni3161* markers, respectively. AA, AB, and BB are represented by 0, 1, and 2, respectively when $y \geq 0.5$ indicates male fish and $y < 0.5$ indicates female fish.
[2] % accuracy = (number of accuracy prediction of fish / the total of fish) x 100.

## DISCUSSION

In this present study, we verified polymorphisms of the sex-linked genetic markers on LG20 and LG23 and assessed their association with phenotypic sex characteristics in red tilapia. Four genetic markers (*Amh*, *SCAR4*, *SCAR5*, and *Oni3161*) were found to be segregating among the Thai red tilapia population.

The results in this study showed that the polymorphism of the *Amh* marker had a significant association with phenotypic sex characteristics of the Thai red tilapia population. This result agreed with previous studies which found the polymorphisms of the *Amh* gene were associated with phenotypic sex characteristics and linked with QTL regions for sex determination in various Nile tilapia populations e.g., Israel, Brazil, Costa Rica, and the Philippines (Eshel et al., 2012; Cáceres et al., 2019). Moreover, it has been reported that the *Amh* gene had the highest overexpression in male compared to female embryos of Nile tilapia at 3 to 7 days postfertilization (Eshel et al., 2012). The *Amh* gene is a member of the transforming growth factor beta (TGF-β) family and is secreted by Sertoli cells and is responsible for the regression of Müllerian duct during male fetal development in mammals, birds, and reptiles (Taslima et al., 2021). Moreover, sequencing analysis of the *Amh* gene in Nile tilapia revealed a male-specific duplication, denoted *Amh*y, differing from the sequence of *Amh* by a 233 bp deletion on exon VII. It is lacking the capability to encode the protein motif that binds to the transforming growth factor beta receptor (TGF-β domain), due to a reading frame shift and disruption of a stop codon (Eshel et al., 2014). Additionally, the *Amh* and *Amh*y had been mapped on LG23 at the QTL region for sex determination of Nile tilapia (Eshel et al., 2014). This evidence suggests that the *Amh* gene may be positively correlated with the sex determination of Nile tilapia.

There was no association of the *SCAR4*, *SCAR5*, and *Oni3161* markers with phenotypic sex characteristics of the Thai red tilapia population. However, the *SCAR4* marker showed a trend toward being associated with phenotypic sex characteristics in the Thai red tilapia with an accuracy of 31.4%. These results are inconsistent with those of previous studies, which have indicated that the *SCAR4* and *SCAR5* markers showed a high association with phenotypic sex in various Chinese Nile tilapia populations with an accuracy of 93-96% (Sun et al., 2014). The *SCAR4* and *SCAR5* markers are sequences characterized amplified region (SCAR) and were developed from amplified fragment length polymorphism (AFLP) markers which are specific to the sex characteristics of Chinese Nile tilapia (Sun et al., 2014). Moreover, the *SCAR4* and *SCAR5* markers had also been mapped on LG23 of Nile tilapia (Sun et al., 2014). Additionally, previous studies have demonstrated that the *Oni3161* marker is significantly associated with sex-determining QTL regions of the Egyptian Nile tilapia population (Palaiokostas et al., 2015; Taslima et al., 2021). The *Oni3161* marker had been localized on LG20 of Nile tilapia (Palaiokostas et al., 2015). These contrasting results might be caused by the different genetic background effects or population structure of those fish populations. Thus, we hypothesized that these genetic markers might be in weak linkage of disequilibrium with QTL regions for sex determination in this Thai red tilapia population.

In the present study, the combination of these two, three, and four markers (*Amh*, *SCAR4*, *SCAR5*, and *Oni3161*) markers showed an increasing association with phenotypic sex characteristics of the red tilapia with an accuracy of 63.0-72.0%. This result is in agreement with previous studies, which demonstrated that the combinations of sex-linked markers on LG1, LG20, and LG23 were associated with QTL regions for sex determination in Nile tilapia populations (Palaiokostas et al., 2015; Taslima et al., 2021). Palaiokostas et al. (2015) reported that allelic combinations of SNP markers on LG1 (rs397507167) and LG20 (*Oni3161*) were highest associated with phenotypic sex in offspring of the Egyptian Nile tilapia population. Similarly, Taslima et al. (2021) reported that allelic combinations of SNP markers on LG1 (*Oni23063*) and LG20 (*Oni3161*) revealed a significant association with phenotypic sex for broodstock of the Egyptian Nile tilapia population. Moreover, allelic combinations of genetic markers on LG1 (*Oni23063*) and LG23 (*Amh*) showed a significant association with phenotypic sex for the progeny of the Egyptian Nile tilapia population (Taslima et al., 2021). The evidence suggested that several sex-linked markers on LG1, LG20, and LG23 are associated with QTL regions for sex determination in Nile tilapia. The sex-determining QTL in Nile tilapia may be regulated by polygenes. These results in this study indicated that the combined sex-linked markers were associated with the phenotypic sex characteristics in the Thai red tilapia population. Therefore, these combined sex-linked markers can be used as a potential tool to detect genetic sex in this red tilapia population, which in turn can be used to optimize methods for producing all-male populations of tilapia (Cáceres et al., 2019). Further studies are needed to affirm the accuracy of these markers in their progeny and various larger fish populations.

## CONCLUSIONS

In the present study, we have verified the polymorphisms of the sex-linked genetic markers on LG20 and LG23 and assessed their association with phenotypic sex characteristics in the Thai red tilapia population. The *Amh* marker showed a significant association with phenotypic sex characteristics of this red tilapia population with an accuracy of 46.2%. There was no association of the *SCAR4*, *SCAR5*, and *Oni3161* markers with phenotypic sex characteristics. However, the combinations of these markers were increasingly associated with the phenotypic sex characteristics of the Thai red tilapia population with an accuracy of 63.0-72.0%. These findings emphasize the importance of these genetic markers could be predicted the phenotypic sex of red tilapias. Therefore, these combined sex-linked markers on LG20 and LG23 may be potential candidate markers for sex determination in the Thai red tilapia population.

## ACKNOWLEDGEMENTS

We would like to thank the Uttaradit Aquaculture Genetics Research and Development Center, Department of Fisheries, Thailand for providing us with the red tilapia samples used in this study. We would also like to thank Julius Rajula, Ph.D. for the English language editing of this manuscript. Furthermore, we wish to thank the anonymous reviewers for their valuable suggestions.

## AUTHOR CONTRIBUTIONS

**Watcharapong Naraballobh;** Methodology, investigation, data curation, formal analysis, writing - original draft, writing - review and editing.
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**Noppasin Sommit;** Investigation, data curation, writing - review and editing.
**Tawatchai Teltathum;** Methodology, investigation, writing - review and editing.
**Hien Van Doan;** Methodology, investigation, writing - review and editing.
**Korawan Sringarm;** Methodology, data curation, writing - review and editing.
**Trisadee Khamlor;** Data curation, formal analysis, writing - review and editing.
**Supamit Mekchay;** Conceptualization, supervision, methodology, investigation, formal analysis, writing - original draft, writing-review and editing, project administration.

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**How to cite this article;**

Watcharapong Naraballobh, Nanthana Pothakam, Worrarak Norseeda, Noppasin Sommit, Tawatchai Teltathum, Hien Van Doan, Korawan Sringarm, Trisadee Khamlor and Supamit Mekchay. Association of genetic markers with sex determination in Thai red tilapia. Veterinary Integrative Sciences. 2022; 20(1): 73 - 83.