DEVELOPMENT OF SEX-SPECIFIC DNA MARKERS IN SNAKESKIN GOURAMI (*Trichopodus pectoralis*) AND THEIR APPLICATION FOR IDENTIFICATION OF NEOMALES

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การพัฒนายีนเครื่องหมายที่จำเพาะต่อเพศในปลาสลิด (*Trichopodus pectoralis*) และการประยุกต์ใช้ในการจำแนกปลานีโอเมล

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาปรัชญาดุษฎีบัณฑิต
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หมิงหัว จาง : การพัฒนายีนเครื่องหมายที่จำเพาะต่อเพศในปลาสลิด (*Trichopodus pectoralis*) และการประยุกต์ใช้ในการจำแนกปลานีโอเมล (DEVELOPMENT OF SEX-SPECIFIC DNA MARKERS IN SNAKESKIN GOURAMI (*Trichopodus pectoralis*) AND THEIR APPLICATION FOR IDENTIFICATION OF NEOMALES). อาจารย์ที่ปรึกษา : ศาสตราจารย์ ดร. สุรินทร บุญอนันธนสาร, 146 หน้า.

คำสำคัญ: ปลาสลิด (*Trichopodus pectoralis*)/การกำหนดเพศ/เครื่องหมาย SNP ที่จำเพาะต่อเพศ/RNA editing/นีโอเมล และนีโอฟีเมล/การผลิตปลาเพศเดียว

ความแตกต่างเพศที่มีผลต่อลักษณะการเจริญเติบโตพบในปลาที่มีความสำคัญทางเศรษฐกิจปลายชนิด ปลาสลิด (*Trichopodus pectoralis*) เป็นปลาที่มีความสำคัญทางเศรษฐกิจมีการเพาะเลี้ยงมากในเอเชียตะวันออกเฉียงใต้ และเป็นปลาที่เพศเมียเจริญเติบโตสูงกว่า ในปัจจุบันได้มีการพัฒนาเทคโนโลยีการแปลงเพศปลาสลิดด้วยฮอร์โมนเอสตราไดออลเพื่อผลิตปลาสลิดเพศเมียล้วนทางการค้า ยังมีทางเลือกของการผลิตปลาสลิดเพศเมียล้วนโดยวิธีทางอ้อมโดยใช้ปลานีโอเมลและการใช้เครื่องหมายโมเลกุลในการจำแนกเพศปลาสลิด จะช่วยเพิ่มประสิทธิภาพของการผลิตปลาสลิดนีโอเมล ซึ่งยังขาดข้อมูลด้านเครื่องหมายโมเลกุลที่มีความจำเพาะต่อเพศปลา ดังนั้นในการศึกษานี้จึงมุ่งพัฒนาเครื่องหมายโมเลกุลที่มีความจำเพาะต่อเพศปลาและประยุกต์ใช้ในการตรวจจีโนไทป์ของปลานีโอเมล

ในการทดลองที่ 1 การวิเคราะห์ทรานสคริปโตมด้วยเทคนิค RNA sequencing ในรังไข่และอัณฑะ พบ RNA SNPs ที่มีความจำเพาะต่อเพศปลา 21 SNPs ผลการวิเคราะห์ลำดับเบสด้วยเทคนิค Sanger และ PACE assays สามารถยืนยัน RNA SNPs ที่มีความจำเพาะต่อเพศปลาจำนวน 9 SNPs โดย SNPs เป็นเฮทเทอโรไซโกตซิตี้ในปลาเพศผู้ และเป็นโฮโมไซโกตซิตี้ในปลาเพศเมีย ผลการศึกษาพบว่า SNP_2, SNP_3, SNP_4, SNP_5, SNP_12, และ SNP_15 อยู่ในยีน *ift172, man2a1, crk, ezh2, cs,* และ *cast* ตามลำดับ อย่างไรก็ตาม SNPs เหล่านี้ไม่มีความจำเพาะต่อเพศปลาเมื่อตรวจวิเคราะห์ในจีโนม ซึ่งอาจเป็นไปได้ว่าเกิดจากกระบวนการ RNA editing ที่มีผลต่อการกำหนดเพศปลา และพบว่ายีน adar ที่เกี่ยวข้องกับกระบวนการ RNA editing มีการแสดงออกสูงในปลาเพศผู้ ซึ่งน่าจะอธิบายได้ว่ากระบวนการ RNA editing เกี่ยวข้องกับการพัฒนาการของเพศปลา นอกจากนี้ SNPs อีก 3 SNPs ได้แก่ SNP_19, SNP_20, และ SNP_21 อยู่บนยีน *kap3l* โดย SNPs ทั้ง 3 มีความจำเพาะต่อเพศปลาทั้งในระดับอาร์เอ็นเอและในจีโนม จึงสามารถนำไปใช้ในการวิเคราะห์เพศปลาในระดับจีโนไทป์ได้ โดยสรุปการศึกษาครั้งนี้ได้พบ SNPs ที่มีความจำเพาะต่อเพศปลาและสามารถนำไปใช้เป็นเครื่องหมายโมเลกุลในการจำแนกเพศปลาได้

ในการทดลองที่ 2 การผลิตปลานีโอเมลโดยการเลี้ยงปลาด้วยอาหารผสมฮอร์โมน 17α-methyltestosterone (MT) ปลาในกลุ่มควบคุมเป็นปลาเพศผู้โดยธรรมชาติ การศึกษาครั้งนี้ได้ใช้

SNP_19, SNP_20, และ SNP_21 ในการจำแนกเพศปลาด้วยเทคนิค Sanger และ PACE assays ผลการศึกษาพบว่า SNPs ทั้ง 3 ให้ผลไปในทางเดียวกันคือ ปลาเพศผู้และปลาเพศผู้ที่ได้รับฮอร์โมน MT มีจีโนไทป์เป็นเฮทเทอโรไซกัส ในขณะที่ปลานีโอเมลและปลาเพศเมียมีจีโนไทป์เป็นโฮโมไซกัส ผลการทดสอบ Progeny testing พบว่าการผสมพันธุ์ระหว่างปลานีโอเมลและปลาเพศเมียให้ลูกปลาเพศเมียทั้งหมด โดยผลการศึกษาครั้งนี้ยืนยันระบบกำหนดเพศ XX/XY ในปลาสลิด ดังนั้น SNPs ที่มีความจำเพาะต่อเพศปลาสามารถนำไปใช้ในการจำแนกปลานีโอเมลได้

ในการทดลองที่ 3 การผลิตปลานีโอฟีเมลโดยการเลี้ยงปลาด้วยอาหารผสมฮอร์โมน 17β-estradiol (E2) โดยการยืนยันผลจีโนไทป์ด้วย SNP_19, SNP_20, และ SNP_21

ผลการศึกษาพบว่าปลาเพศเมียที่ได้รับฮอร์โมน E2 มีจีโนไทป์เป็นโฮโมไซกัส ผลการทดสอบ Progeny testing พบว่าการผสมพันธุ์ระหว่างปลานีโอฟีเมลและปลาเพศผู้ให้ลูกปลาเพศผู้ 73.34 ± 3.16%ซึ่งผลการศึกษาครั้งนี้ยืนยันระบบกำหนดเพศ XX/XY ในปลาสลิด ดังนั้น SNPs ที่มีความจำเพาะต่อเพศปลาสามารถนำไปใช้ในการจำแนกปลานีโอฟีเมลได้เช่นกัน

โดยสรุป ดุษฎีนิพนธ์ฉบับนี้ได้พัฒนา SNPs ที่มีความจำเพาะต่อเพศ ที่สามารถนำมาใช้ในการจำแนกเพศปลา การที่ปลาเพศผู้แสดงเฮทเทอโรไซกัสของ SNPs สามารถยืนยันระบบกำหนดเพศในปลาสลิดเป็นระบบ XX/XY และ SNPs ที่มีความจำเพาะต่อเพศปลานี้สามารถนำไปใช้ในการจำแนกปลานีโอเมลและนีโอฟีเมล

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Keyword: SNAKESKIN GOURAMI (*Trichopodus pectoralis*)/SEX DETERMINATION/SEX-SPECIFIC SNP MARKERS/RNA EDITING/NEOMALE AND NEOFEMALE/MONOSEX PRODUCTION

Sexual dimorphism in growth characteristics is observed in many economically important fish species. The snakeskin gourami (*Trichopodus pectoralis*), an economically important freshwater fish widely cultured in Southeast Asia, exhibits pronounced female-biased growth. Recently, estradiol-induced sex reversal technology has been established to produce all-female populations for commercial purposes. In addition, an indirect method to generate neomales could be an alternative strategy to produce all-female fish populations, and a genotypic sex-specific marker could offer an efficient procedure to produce neomales. However, information on sex-specific markers in snakeskin gourami remains limited. Therefore, this study aimed to develop sex-specific markers and apply them to determine the genotype of neomales.

In Experiment I, transcriptome-based SNP analysis using RNA sequencing of ovarian and testicular tissues identified 21 candidate sex-specific RNA SNPs. Validation by Sanger sequencing and PACE assays confirmed nine sex-specific SNPs identified from RNA-seq, that were heterozygous in males and homozygous in females. Among these SNPs, six SNPs including SNP_2, SNP_3, SNP_4, SNP_5, SNP_12, and SNP_15 were located in *ift172, man2a1, crk, ezh2, cs,* and *cast*, respectively. However, these SNPs were not detected in genomic DNA, suggesting that RNA editing may be involved in sex determination. Additionally, male-biased expression of *adar* suggested that RNA editing may contribute to sex differentiation in snakeskin gourami. The remaining three SNPs (SNP_19, SNP_20, and SNP_21) were located in *kap3l*. Subsequent validation at the genomic DNA level showed that SNP_19, SNP_20, and SNP_21 were concordant with nucleotide variation in genomic DNA, suggesting that these three SNPs could be

used for sex genotyping. Collectively, these findings demonstrate the identification of sex-specific SNP markers for sex determination in snakeskin gourami.

In Experiment II, neomale broodstock were generated through dietary administration of 17α-methyltestosterone (MT), while the control group consisted of naturally developed broodstock. Genetic sex was determined using sex-specific SNP markers (SNP_19, SNP_20, and SNP_21) via Sanger sequencing and PACE SNP assays. All markers exhibited consistent genotyping patterns, with MT-treated males and control males showing heterozygous genotypes, whereas MT-derived neomales and females displayed homozygous genotypes. Progeny testing confirmed that crosses between neomales (XX) and females (XX) consistently produced 100% female offspring. These results support an XX/XY sex-determination system in snakeskin gourami. Therefore, sex-specific SNP markers could be applied to identify neomales.

In Experiment III, neofemales induced by 17β-estradiol (E2) were genetically confirmed using SNP_19, SNP_20, and SNP_21. E2-treated neofemales and control males exhibited heterozygous genotypes, whereas E2-treated females displayed homozygous genotypes. Crosses between neofemales (XY) and males (XY) produced male-biased progeny (male: 73.34 ± 3.16%), consistent with an XX/XY sex determination system. Therefore, sex-specific SNP markers could also be applied to identify neofemales.

In summary, this study developed reliable sex-specific SNP markers and demonstrated their application in genetic sex identification. The presence of heterozygous SNPs in males suggests an XX/XY sex-determination system in snakeskin gourami. These sex-specific SNP markers can be applied for genotyping not only neomales but also neofemales.

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LIST OF ABBREVIATIONS

ADAR	=	Adenosine deaminase acting on RNA
AFLP	=	Amplified fragment length polymorphism
Amh	=	Anti-Müllerian hormone
AO	=	Adult ovary
AT	=	Adult testis
Bp	=	base pair
C	=	Control group
Cast	=	calpastatin
CDS	=	Coding DNA sequence
cDNA	=	Complementary DNA
crk	=	CRK proto-oncogene, adaptor protein
cs	=	Citrate synthase
DArTseq	=	Diversity Arrays Technology sequencing
ddRAD	=	Double-digest restriction-site associated DNA sequencing
DEG	=	Differentially expressed gene
DNA	=	Deoxyribonucleic acid
DO	=	Dissolved oxygen
dph	=	Days post-hatching
E2	=	17β-estradiol
ESD	=	Environmental sex determination
ezh2	=	Enhancer of zeste homolog 2
F	=	ES-treated female
FAO	=	Food and Agriculture Organization of the United Nations
FASTQ	=	Format for sequencing reads
FISH	=	Fluorescent in situ hybridization
FSH	=	Follicle-stimulating hormone
GATK	=	Genome Analysis Toolkit

LIST OF ABBREVIATIONS (Continued)

GBS	=	Genotyping-by-sequencing
gDNA	=	Genomic DNA
GSD	=	Genotypic sex determination
GSI	=	Gonadosomatic index
GWAS	=	Genome-wide association study
H&E	=	Hematoxylin and eosin
HRM	=	High-resolution melting
ift172	=	Intraflagellar transport protein 172
indel	=	Insertion/deletion polymorphism
JO	=	Juvenile ovary
JT	=	Juvenile testis
kap3l	=	Kinesin-associated protein 3-like
KASP	=	Kompetitive allele-specific PCR
LHRHa	=	Luteinizing hormone-releasing hormone analog
man2a1	=	mannosidase, alpha, class 2A, member 1
MO	=	Mature oocytes
MS-222	=	Tricaine methanesulfonate
MT	=	17α-methyltestosterone
N	=	ES-treated neofemale
NGS	=	Next-generation sequencing
NTC	=	No-template control
OG	=	Oogonia
PACE	=	PCR Allele Competitive Extension
PCR	=	Polymerase chain reaction
PSC	=	Primary spermatocytes
PVO	=	Previtellogenic oocytes
RAD-seq	=	Restriction-site associated DNA sequencing
RAPD	=	Random amplified polymorphic DNA
RIN	=	RNA integrity number

LIST OF ABBREVIATIONS (Continued)

RNA-seq	=	RNA sequencing
RT-PCR	=	Reverse transcription polymerase chain reaction
S	=	Supermale group
SAM	=	Sequence alignment/map
SD	=	Sexual dimorphism
SG	=	Spermatogonia
SNP	=	Single nucleotide polymorphism
SSC	=	Secondary spermatocytes
SSR	=	Simple sequence repeats
ST	=	Spermatids
SUT	=	Suranaree University of Technology
T7EI	=	T7 endonuclease I
TCA	=	Tricarboxylic acid
Tm (°C)	=	Annealing temperature (°C)
TSD	=	Temperature-dependent sex determination
UTR	=	Untranslated region
WT	=	Wild type
XX	=	Female genotype
XY	=	Male genotype
YY	=	Supermale genotype
ZW	=	Female heterogametic sex chromosome system
ZZ	=	Male homogametic sex chromosome system
χ^2	=	Chi-square test

CHAPTER I

INTRODUCTION

1.1 Introduction

Sex determination in teleost fishes exhibits remarkable diversity and complexity compared with that in other vertebrates. Unlike mammals and birds, in which sex is typically governed by highly conserved sex chromosome systems, fishes display a wide variety of sex determination patterns that differ markedly among taxa (Devlin and Nagahama, 2002; Mank and Avise, 2009). These systems include environmental sex determination (ESD), in which sex differentiation can be influenced by external environmental conditions, resulting in variable sex ratios among populations. In contrast, under genetic sex determination (GSD), sexual fate is primarily determined by sex chromosomes or sex-linked genomic loci, including simple systems such as XX/XY and ZZ/ZW, as well as multiple sex chromosome systems (Devlin and Nagahama, 2002; Mank and Avise, 2009). In addition, mixed mechanisms have been reported in which genetic factors and environmental cues may jointly influence sexual development (Baroiller and D'Cotta, 2001; Baroiller et al., 2009). However, in many teleost fishes, sex chromosomes are often poorly differentiated or cytogenetically indistinguishable, which limits the applicability of morphological and classical cytogenetic approaches for genetic sex identification (Mank and Avise, 2009). Under such conditions, the development of genetic markers provides an effective solution for accurately determining genetic sex (Mei and Gui, 2015).

In teleost fishes, distinct heteromorphic chromosomes are rarely observed (Cioffi et al., 2017). Instead, genetic markers derived from sex-associated genomic regions provide an effective approach for genetic sex identification, enabling reliable discrimination at the DNA level across developmental stages. These sex-linked markers have been widely applied in studies of sex chromosome systems and aquaculture breeding programs, particularly for early sex identification and monosex population production (Liu and Cordes, 2004; Luckenbach et al., 2017; Mei and Gui, 2015; Lu et al., 2024).

A wide range of molecular marker systems has been developed for genetic analysis in fishes, including randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeats (SSR), and insertion–deletion polymorphisms (InDels) (Liu and Cordes, 2004; Mei and Gui, 2015). These markers have been successfully applied in population genetics and sex-linked marker development. However, limitations such as low reproducibility, dominant inheritance, or restricted genomic coverage may reduce their efficiency in large-scale applications. In recent years, single-nucleotide polymorphisms (SNPs) have emerged as the most widely used genetic markers in fish studies. Owing to their high abundance throughout the genome, high genetic stability, and compatibility with high-throughput genotyping platforms, SNP markers provide a reliable and cost-effective approach for genetic sex identification and breeding-related analyses (Mei and Gui, 2015; Zhao et al., 2019; Liu et al., 2018). Consequently, transcriptome-based SNP discovery has become an effective strategy for identifying sex-linked loci in non-model aquaculture species.

The snakeskin gourami (*Trichopodus pectoralis*) is an economically important freshwater fish widely cultured in Southeast Asia (Klinmalai et al., 2021; Panthum et al., 2021; Dinh-Hung et al., 2023). This species exhibits pronounced sexual size dimorphism, with females showing faster growth and larger body size than males, resulting in higher commercial value (Boonanuntanasarn et al., 2020; Kabpha et al., 2023). However, the sex determination mechanism of snakeskin gourami (*T. pectoralis*) remains poorly understood, and reliable methods for early genetic sex identification are currently lacking (Panthum et al., 2021; Jantawongsri et al., 2025). These limitations highlight the necessity for developing robust molecular markers to enable precise genetic sex identification in this species. The present study aimed to develop reliable sex-specific DNA markers in snakeskin gourami using transcriptome sequencing and SNP validation. By identifying and verifying sex-linked SNP loci, this study seeks to establish an effective molecular approach for genetic sex identification, thereby providing a scientific basis for broodstock management and the potential development of sustainable monosex breeding strategies.

1.2 Research objectives

1.2.1 To develop sex-specific DNA markers and validate them for identifying the genotypic sex of snakeskin gourami through transcriptome-based SNP discovery.

1.2.2 To apply the developed sex-specific markers to identify the genotypic sex of hormone-induced neomales or neofemales.

1.3 Research hypotheses

1.3.1 There are consistent and heritable differences at the nucleotide level (e.g., SNPs) between genetic males and females in *Trichopodus pectoralis*. Consequently, sex-linked SNPs can be developed into reliable DNA markers for identifying the genotypic sex in snakeskin gourami.

1.3.2 The sex determination system in snakeskin gourami is, at least in part, controlled by genetic factors. Therefore, the identified sex-specific DNA markers can be used to determine the genotypic sex of neomales (phenotypic males induced via hormone treatment) or neofemales (phenotypic females induced via hormone treatment).

1.4 Scope of the study

This study focuses on the development of sex-specific markers using transcriptome-based discovery at the transcriptional level. To ensure accurate sex determination, the collected gonads were divided into two parts: one portion was preserved at –80°C for total RNA extraction, while the other underwent histological examination to confirm phenotypic sex and developmental stage.

To develop reliable sex-specific markers, transcriptome information from testes and ovaries of juvenile and adult fish was used. Comparative analysis of male and female transcriptomes from both juvenile and adult stages enabled the identification of candidate sex-linked genetic markers associated with genotypic sex. Following bioinformatic filtering, selected candidate sex-specific RNA SNPs were validated in another fish population. In addition, these candidate RNA SNPs were validated in genomic DNA to confirm their potential as sex-specific markers.

PCR-based genotyping assays were developed to assess the presence and sex-

association of these markers in a broader population. Once validated, these molecular markers were applied to identify the genotypic sex of hormonally induced neomales (phenotypic males with a presumptive XX genotype). Neomales were crossed with normal females, and the resulting offspring were sexed to determine sex ratios. Similarly, the molecular markers were used to identify the genotypic sex of hormonally induced neofemales (phenotypic females with a presumptive XY genotype). Neofemales were crossed with normal males, and the resulting offspring were sexed to determine sex ratios. Overall, this study aims to establish a robust, non-invasive, and reliable method for sex identification, which could support the production of monosex populations in *T. pectoralis* aquaculture, thereby enhancing production efficiency and economic value.

1.5 Expected benefits

The development of SNP-based molecular markers would enable precise identification of the genetic sex of *T. pectoralis*. This technology would allow for early sex determination, particularly during developmental stages when fish have not yet exhibited sex-related morphological differences. Therefore, this study aims to establish a robust, non-invasive, and reliable method for sex identification, which could support the production of monosex populations in *T. pectoralis* aquaculture.

Since female *T. pectoralis* grow faster than males, all-female production is preferred in fish farming. Recently, 17-β estradiol-induced sex reversal has been implemented to produce all-female populations. To directly generate all-female populations without hormone use, neomales are required. Neomales can be produced through dietary supplementation with 17-α methyltestosterone, and progeny testing is typically used to identify their genotypic sex. However, this approach is time-consuming, labor-intensive, and requires large hatchery facilities.

The application of sex-specific markers would allow for rapid, non-labor-intensive, and facility-efficient genotypic sex determination, enabling more efficient production of neomale broodstock. These neomales can be selectively mated with normal females to produce all-female progeny, which typically exhibit faster growth and higher market value.

This hormone-free strategy offers a sustainable, consumer-friendly, and

environmentally safe alternative to traditional hormone-based sex reversal methods, thereby enhancing production efficiency in *T. pectoralis* aquaculture. Furthermore, this study contributes critical knowledge toward understanding the genetic basis of sex differentiation and supports the long-term objective of developing stable, monosex breeding programs. Overall, the findings from this study are expected to provide both practical applications for the aquaculture industry and scientific insights into the molecular mechanisms of sex determination in teleost fishes, laying a foundation for future genetic and reproductive studies in *T. pectoralis*.

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CHAPTER II

LITERATURE REVIEW

2.1 Biological Characteristics of Snakeskin Gourami

The snakeskin gourami (*Trichopodus pectoralis*) is a freshwater teleost belonging to the family Osphronemidae (Figure 2.1 and Table 2.1), which also includes other labyrinth fishes such as the giant gourami (*Osphronemus goramy*) and the three-spot gourami (*Trichopodus trichopterus*). *T. pectoralis* is one of the most economically important gourami species in Southeast Asia and is widely cultured for both food consumption and ornamental purposes (Nakharuthai et al., 2022). Its natural range extends across Thailand, Cambodia, Vietnam, Laos, Malaysia, and Indonesia, with introductions into Myanmar, Sri Lanka, and India to support aquaculture (Dinh-Hung et al., 2023; Klinmalai et al., 2021; Ramadhani et al., 2020; *The State of World Fisheries and Aquaculture 2020*, 2020; Vromant et al., 2001).

Figure 2.1 Snakeskin gourami (*Trichopodus pectoralis*).

Table 2.1 Taxonomy of the snakeskin gourami (*Trichopodus pectoralis*).

Rank	Classification
Phylum	Chordata
Class	Actinopterygii
Order	Anabantiformes
Family	Osphronemidae
Genus	*Trichopodus*
Species	*Trichopodus pectoralis*

Reference: Regan, 1909.

In Thailand, the snakeskin gourami has long been integrated into rice–fish farming systems, supported by its strong tolerance to hypoxic conditions and broad ecological adaptability (Ninwichian et al., 2018). The ecological success of *T. pectoralis* is largely attributed to the labyrinth organ, a suprabranchial structure that enables aerial respiration, allowing the species to thrive in oxygen-poor habitats such as swamps, floodplains, and rice paddies (Martin and Graham, 1998; Froese, 2006). Together with its omnivorous feeding habits, ranging from zooplankton and insect larvae to algae and detritus, these traits promote stable growth in both natural and cultured environments and make the species highly compatible with integrated rice–fish farming systems (Panthum et al., 2021).

Beyond its value as a food fish, *T. pectoralis* also holds cultural and economic importance in Thailand, where dried and salted gourami fillets are considered a traditional delicacy, and its robust physiology and distinctive body patterning support demand in the ornamental fish trade (Klinmalai et al., 2021). However, rapid urbanization and the reduction of agricultural land have constrained traditional extensive production systems, while increasing consumer demand, particularly in Thailand, where *T. pectoralis* consistently ranks among the top five farmed freshwater fishes, has driven a transition toward semi-intensive and intensive aquaculture practices (Boonanuntanasarn et al., 2020; Kabpha et al., 2023). Despite these developments, current production levels remain insufficient to satisfy both domestic consumption and export markets (Dinh-Hung et al., 2023).

Snakeskin gourami is a gonochoristic species exhibiting clear sexual size

dimorphism, in which females generally grow faster and attain a larger body size than males (Boonanuntanasarn et al., 2020; Kabpha et al., 2023). Under culture conditions, females have been reported to reach harvest size approximately 20–30% larger than males, making female-biased populations economically advantageous in aquaculture production (Boonanuntanasarn et al., 2020; Kabpha et al., 2023). This female-biased growth advantage highlights the potential value of an all-female culture in aquaculture profitability. Yet, the genetic and environmental mechanisms underpinning sex determination remain poorly understood. Like many teleosts, *T. pectoralis* may possess homomorphic sex chromosomes that cannot be distinguished cytogenetically, complicating efforts to manipulate sex for production purposes (Araya-Jaime et al., 2017; Artoni et al., 2015). These knowledge gaps have hindered selective breeding programs, underscoring the need for molecular approaches to address them. Recent advances in genomics and transcriptomics have begun to provide tools for sex identification and genetic improvement. Subsequent sections of this review will explore these developments in the broader context of fish aquaculture.

2.2 Sex determination system in fish

Sex determination mechanisms in teleost fishes are remarkably diverse, ranging from classical male-heterogametic (XY) and female-heterogametic (ZW) systems to more complex arrangements (Fang et al., 2025). These systems often differ not only among families but also among closely related species. The identification of these systems has relied on a variety of cytogenetic, molecular, and genomic approaches, including karyotyping, fluorescent in situ hybridization (FISH), microsatellite-based linkage mapping, high-density single-nucleotide polymorphisms (SNPs) genotyping, and high-throughput sequencing technologies (e.g., RAD-seq, GWAS, and whole-genome assembly) (Devlin and Nagahama, 2002). To provide an overview of the breadth of sex determination diversity and the tools used to study it, Table 2.2 summarizes representative teleost species, their documented sex determination systems, the methods used for their characterization, and corresponding key references. This comparative framework highlights the complexity of sex determination across fishes and underscores the need for integrative molecular tools to support monosex breeding and sex-control strategies in aquaculture.

Two broad categories are often distinguished: genotypic sex determination (GSD) and environmental sex determination (ESD). In GSD systems, sex is primarily defined by alleles at a sex-determining locus, and the classic heterogametic models XX/XY (male heterogamety) and ZZ/ZW (female heterogamety) are both widely represented in teleosts (Mank and Avise, 2009). However, more complex systems exist, including multiple sex chromosome arrangements such as X_1X_2Y, ZW_1W_2, or X0/XX, as reported in several cyprinids and flatfishes (Kitano and Peichel, 2012). In contrast, ESD occurs when abiotic or social cues determine phenotypic sex, regardless of the genotype. Temperature is the most influential factor, with temperature-dependent sex determination widely reported in both aquaculture and wild populations (Baroiller and D'Cotta, 2001; Devlin and Nagahama, 2002). Other environmental variables, such as pH, photoperiod, or density, have also been shown to bias sex ratios in some taxa (Reddon and Hurd, 2013). Importantly, mixed systems (GSD + ESD) occur in many species, where genetic cues establish a predisposition that can be overridden by environmental signals (Sandra and Norma, 2010).

Cytogenetic approaches, which visualize heteromorphic chromosomes through karyotyping or FISH, have proven effective in species with highly differentiated sex chromosomes, such as tilapia and medaka (Harvey et al., 2002; Matsuda et al., 1998). However, for most fish with homomorphic chromosomes, these methods provide little insight (Charlesworth and Mank, 2010). Breeding experiments, often involving sex-reversed individuals, can help reveal patterns of inheritance, but such approaches are labor-intensive, time-consuming, and limited to a handful of model or commercial species (Piferrer, 2001).

Molecular approaches are increasingly filling this gap by offering a powerful means to detect sex-linked loci in species lacking cytogenetic signals. Association mapping, linkage analysis, and high-throughput sequencing technologies have revealed small regions of suppressed recombination associated with sex in a wide range of fishes (Bao et al., 2019). These findings highlight that sex chromosomes in teleosts are often young and dynamic, with frequent turnovers and rapid divergence across lineages (Bachtrog et al., 2014; Kitano and Peichel, 2012). For aquaculture species such as snakeskin gourami, molecular investigations are essential to establish the underlying heterogametic system and to guide the design of reliable sex-control strategies.

Although extensive progress has been made in elucidating sex determination systems in diverse teleosts, the underlying mechanism in snakeskin gourami remains unresolved, highlighting the need for species-specific molecular investigations.

Table 2.2 Sex determination systems and identification approaches in teleosts.

No.	Sex Determination System	Identification Method	Species	Reference
1	XX/XY	FISH	Medaka (*Oryzias latipes*)	Matsuda et al. (2002)
2	ZZ/ZW	Linkage mapping	Tanganyika tilapia (*Oreochromis tanganicae*)	Cnaani and Kocher (2008)
3	$X_1X_1X_2X_2$ / X_1X_2Y	Cytogenetics; GWAS	Collichthys lucidus (*Collichthys lucidus*)	Cai et al. (2019)
4	ZZ/ZW	GWAS, SNP	Chilean Jack Mackerel (*Trachurus murphyi*)	Canales-Aguirre et al. (2025)
5	XX/XY	Genome Walking	Silver carp (*Hypophthalmichthys molitrix*)	Liu et al. (2018)
6	ZZ-ZW_1W_2	Molecular Cytogenetics	Lizardfish (*Synodontidae*)	Ueno et al. (2001)
7	$X_1X_1X_2X_2$/X_1X_2Y	Conventional Cytogenetics	Freshwater stingrays (*Potamotrygonidae*)	Cruz et al. (2011)
8	ZZ/ZW	GWAS	Determination of turbot (*Scophthalmus maximus*)	Martínez et al. (2021)
9	ZZ/ZW	Molecular marker	Clearhead icefish (*Protosalanx hyalocranius*)	Xing et al., (2021)
10	XX/XY_1Y_2	Conventional cytogenetics	Ancistrus sp.1 "Balbina"	De Oliveira et al. (2008)
11	$Z_1Z_1Z_2Z_2$ / $Z_1Z_2W_1W_2$	Conventional cytogenetics	Ancistrus sp.2 "Barcelos"	De Oliveira et al. 2008)
12	$X_1X_1X_2X_2$ / X_1X_2Y	Molecular cytogenetics (FISH)	Gasterosteus aculeatus (*Japanese Population*)	Kitano and Peichel (2012)
13	$X_1X_1X_2X_2$/$X_1Y_1X_2Y_2$	Conventional & Molecular Cytogenetics	Bunocephalus Coracoideus (*Aspredinidae*)	Ferreira et al. (2016)
14	ZZ/Z0	Molecular cytogenetics	Glass Knifefish (*Eigenmannia trilineata*)	Araya-Jaime et al. (2017)
15	XX/X0	Conventional Cytogenetics	Armored catfish (*Loricariidae: Hypostominae*)	Alves et al. (2006)

2.3 Molecular Markers for Sex Determination in Teleost Fishes

Molecular markers have fundamentally advanced the study of sex determination in fishes and provided practical tools for aquaculture. By enabling early and reliable identification of genetic sex, these markers facilitate broodstock management, allow the detection of sex-reversed individuals, and support the production of monosex populations without reliance on hormonal treatments (Mei

and Gui, 2015). Early studies primarily employed low-throughput molecular marker systems. For example, random amplified polymorphic DNA (RAPD), a PCR-based technique using arbitrary primers to amplify random genomic regions, was applied to identify sex-associated markers in common carp (*Cyprinus carpio*) (Chen et al., 2009). Amplified fragment length polymorphism (AFLP), which combines restriction enzyme digestion with selective PCR amplification to generate genome-wide polymorphic profiles, was successfully used in rainbow trout (*Oncorhynchus mykiss*) (Felip et al., 2005). In addition, simple sequence repeats (SSR), co-dominant markers based on short tandem DNA repeats with high polymorphism and reproducibility, have been applied for sex-linked marker identification in half-smooth tongue sole (*Cynoglossus semilaevis*) (Song et al., 2012). Although these approaches contributed to the initial discovery of sex-linked loci in several species, their broader application has been constrained by labor-intensive procedures, limited genomic coverage, and relatively low resolution.

The advent of next-generation sequencing (NGS) has revolutionized marker discovery, allowing genome-wide identification of SNPs and insertion/deletion polymorphisms (indels) (Mei and Gui, 2015; Yano et al., 2012). Reduced-representation sequencing methods such as RAD-seq and ddRAD have been particularly useful in non-model fishes, facilitating dense marker development at moderate cost (Andrews et al., 2016). Moreover, RNA sequencing (RNA-seq) has emerged as a complementary approach, especially in species with homomorphic sex chromosomes, by revealing sex-biased gene expression and coding SNPs that can serve as candidate sex-specific markers (Qian et al., 2014). Table 2.3 summarizes representative teleost species in which transcriptomic (RNA-seq) and/or genome-wide sequencing approaches have contributed to the discovery and validation of sex-specific DNA markers.

Transcriptome-based approaches have also been widely applied for SNP discovery and marker validation in reptiles and amphibians, demonstrating their effectiveness in identifying reliable genetic markers beyond teleost fishes. Although RNA-seq typically generates a large number of putative variants, subsequent filtering and validation can yield highly robust SNP markers suitable for genetic and breeding-related applications. Similar transcriptome-derived SNP strategies have been successfully implemented in livestock and plant species, further highlighting the broad applicability and reliability of this approach (Ma et al., 2016; Pierzchała et al., 2019).

Collectively, these findings indicate that transcriptome-based SNP discovery provides a powerful and versatile framework for genetic marker development, particularly in non-model species with poorly characterized genomes. Accordingly, SNP markers were selected in the present study as the primary strategy for developing reliable sex-specific DNA markers in snakeskin gourami.

In snakeskin gourami, transcriptomic analyses have revealed pronounced differences in gene expression between testes and ovaries, providing important insights into gonadal development and sexual differentiation (Boonanuntanasarn et al., 2020; Kabpha et al., 2023). Recent studies have further identified sex-biased gene expression patterns in both juvenile and adult gonads, highlighting candidate genes potentially involved in steroidogenesis, germ cell development, and gonadal differentiation pathways (Boonanuntanasarn et al., 2020; Jantawongsri et al., 2025). These findings suggest that transcriptomic resources offer a valuable foundation for exploring the genetic basis of sex differentiation in this species.

Importantly, SNPs located within sex-biased genes or in linkage disequilibrium with sex-determining regions represent promising molecular markers for genetic sex identification. However, transcriptome-based discovery alone is insufficient without systematic validation, as sex-biased expression may reflect developmental stage rather than underlying genetic sex. Therefore, distinguishing genetic sex–associated SNPs from stage-dependent expression patterns is essential for developing reliable and stable DNA markers applicable across developmental stages (Leet et al., 2013).

Despite increasing transcriptomic knowledge, integrated studies combining RNA-seq–based SNP discovery with marker validation in phenotypically confirmed individuals remain limited in *T. pectoralis*. A systematic approach involving SNP identification from gonadal transcriptomes, followed by validation using PCR-based genotyping in independent populations, is therefore required to establish robust sex-specific DNA markers and to clarify the genetic architecture underlying sex determination in this species (Zhao et al., 2019).

Table 2.3 Sex-specific molecular markers identified in teleost fishes using sequencing-based approaches.

No.	Species (Latin name)	Transcriptomic / Sequencing method	Type of molecular marker	Reference
1	Northern Snakeheads (*Channa argus*)	RNA-Seq + GWAS	Sex-specific SNP & InDel	Liu, H et al. (2024)
2	Hypophthalmichthys nobilis (*bighead carp*)	2b-RAD + genome-wide sequencing	Male-specific DNA marker	Liu, H et al. (2018)
3	silver carp (*Hypophthalmichthys molitrix*)	2b-RAD + genome-wide sequencing	Male-specific DNA marker	Liu, H et al. (2018)
4	Japanese meagre (*Argyrosomus japonicus*)	PacBio HiFi + Hi-C genome assembly + GWAS of sex	Sex-linked DNA markers	Liu, Y et al. (2024)
5	clearhead icefish (*Protosalanx hyalocranius*)	Whole-genome resequencing	Female-specific DNA marker (deletion)	Xing et al. (2021)
6	blunt snout bream (*Megalobrama amblycephala*)	WGS	Male-specific DNA marker	Fu et al. (2024)
7	channel catfish (*Ictalurus punctatus*)	Y-chromosome sequencing + comparative RNA-seq	Sex-specific isoform / DNA marker	Bao et al. (2019)
8	Crimson seabream (*Pargyrops edita*)	Transcriptomic (RNA-seq)	Simple sequence repeats (SSRs)	Shan et al. (2021)
9	European sea bass (*Dicentrarchus labrax*)	Small RNA-seq (miRNA sequencing)	sex-biased MiRNA markers	Van Gelderen et al. (2025)
10	darkbarbel catfish (*Pelteobagrus vachelli*)	GWAS and transcriptome	male sex-specific markers	Zhang et al. (2026)
11	Chinese tongue sole (*Cynoglossus semilaevis*)	Genome/Genotyping assay	Female-specific structural variation (Indel) & SNPs	Zhang et al. (2019)
12	yellow catfish (*Pelteobagrus fulvidraco*)	Transcriptomic (454 GS-FLX sequencing)	SNPs, SSRs, and INDELs from transcriptome	Chen et al., (2015)

2.4 Sex reversal and monosex breeding strategies in aquaculture

Sex reversal has long been applied in aquaculture as an effective strategy to generate monosex populations that maximize sex-specific growth advantages. Due to the pronounced sexual plasticity of teleost fish, sexual differentiation can be redirected during early developmental stages through endocrine manipulation, allowing to produce phenotypic males or females regardless of their genetic sex (Devlin and Nagahama, 2002). This biological flexibility provides the theoretical foundation for monosex breeding programs aimed at improving production efficiency, uniformity, and economic return.

At the physiological level, endocrine regulation plays a central role in directing gonadal differentiation and constitutes the mechanistic basis of artificial sex reversal.

Suppression of aromatase (cyp19a1a), the key enzyme responsible for estrogen biosynthesis, promotes testicular development, whereas elevated aromatase activity favors ovarian differentiation (Guiguen et al., 2010; Lambeth et al., 2013). Male-associated pathways involving anti-Müllerian hormone (Amh) and gonadotropins such as follicle-stimulating hormone (FSH) further modulate sex differentiation in a stage- and dose-dependent manner (Huang et al., 2019; Zhou et al., 2019). In addition, stress-related endocrine factors, including cortisol, may interact with these hormonal pathways and influence sexual outcomes under specific rearing conditions (Shen and Wang, 2014).

In practical aquaculture, sex reversal is commonly achieved through dietary or immersion treatments using steroid hormones, most notably 17β-estradiol (E2) for feminization and 17α-methyltestosterone (MT) for masculinization (Kabpha et al., 2023; Mu et al., 2024). Based on the mode of application, monosex production strategies are generally classified as direct or indirect. As illustrated in Figures 2.2 and 2.3, direct methods involve hormonal treatment of larvae during the labile period of gonadal differentiation, resulting in phenotypic males or females in the grow-out population. Indirect strategies, in contrast, rely on the generation of sex-reversed broodstock (neomales or neofemales), which are subsequently crossed with normal individuals to produce genetically uniform monosex offspring without hormonal exposure of the commercial generation.

Among these approaches, indirect monosex breeding has gained increasing attention because it mitigates concerns related to hormone residues, environmental contamination, and consumer acceptance. Figure 2.2 summarizes the pathways for monosex female production, in which androgen-induced neomales (phenotypic males with an XX genotype) are crossed with normal females to generate all-female progeny. Similar principles apply to all-male production systems using neofemales (Figure 2.3). These broodstock-based strategies enable sustainable monosex production while maintaining genetic stability across generations.

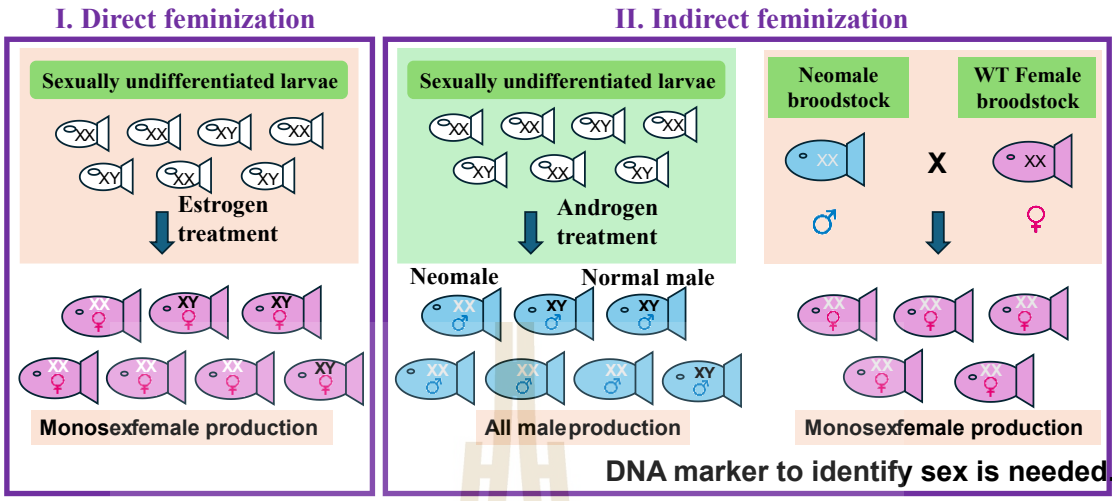

Figure 2.2 Schematic illustration of direct and indirect feminization strategies for monosex female production in fish.

The diagram illustrates two approaches for producing viable all-female populations. In direct feminization, sexually undifferentiated larvae are treated with estrogen, resulting in a phenotypically female population. In indirect feminization, androgen treatment during sexually undifferentiated larvae induces sex reversal, producing neomales that exhibit male phenotype but retain a female (XX) genotype. Neomale broodstock are crossed with wild-type females, leading to the production of monosex female offspring without hormonal treatment of the progeny. The figure highlights the use of early developmental stages and sex-reversed broodstock for female-biased aquaculture production.

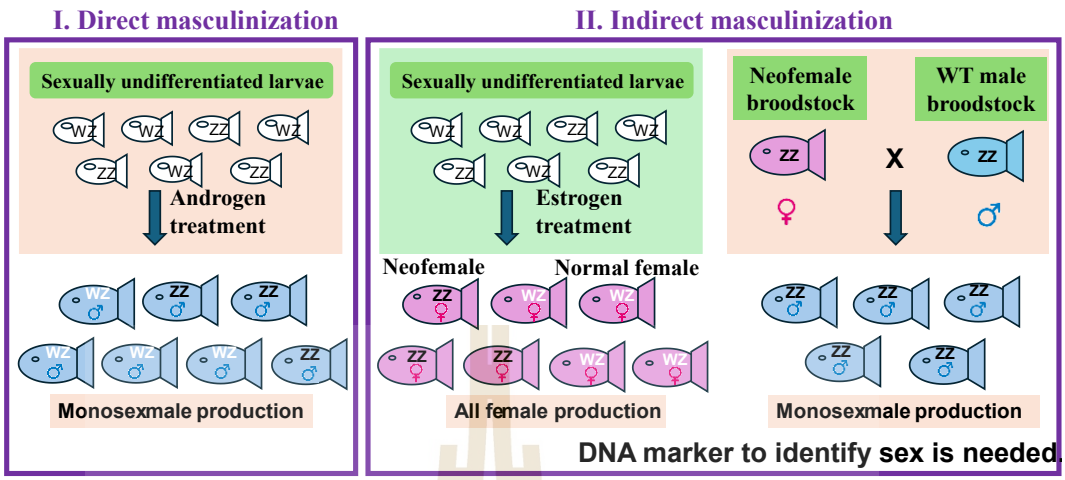

Figure 2.3 Schematic illustration of direct and indirect masculinization strategies for monosex male production in fish.

The figure shows the procedures used to obtain viable monosex populations through hormonal manipulation and broodstock-based approaches. In direct masculinization, sexually undifferentiated larvae are treated with androgen, resulting in phenotypic male populations. In indirect masculinization, estrogen treatment of the sexually undifferentiated larvae induces sex reversal, producing neofemales that exhibit a female phenotype but retain a male (ZZ) genotype. Neofemale broodstock are crossed with wild-type males, producing viable all-male offspring. The diagram highlights the use of early developmental stages and sex-reversed individuals for efficient monosex production in aquaculture.

The economic importance of monosex breeding is closely linked to sexual dimorphism in growth and performance traits. In many aquaculture species, including snakeskin gourami, half-smooth tongue sole (*Cynoglossus semilaevis*), largemouth bass (*Micropterus salmoides*), mandarin fish (*Siniperca chuatsi*), and sablefish (*Anoplopoma fimbria*), females generally exhibit faster growth and attain larger body size, making female-biased populations particularly advantageous (Bi et al., 2020; Kabpha et al., 2023; Luckenbach et al., 2017; Zhou and Chen, 2016). Conversely, male-biased growth has been reported in species such as northern snakehead (*Channa argus*), zig-zag eel (*Mastacembelus armatus*), and Nile tilapia (*Oreochromis* spp.), where all-male culture systems are preferred (Asad et al., 2024; Lu et al., 2024; Wang et al., 2019). Beyond

growth performance, sexual dimorphism may also affect ornamental traits, disease resistance, and stress tolerance, further shaping production strategies under intensive farming conditions (Eliason et al., 2020; Fjelldal et al., 2023; Morris et al., 2020).

Despite the proven effectiveness of sex reversal and monosex breeding, the long-term sustainability of these systems relies heavily on accurate identification of genetic sex and reliable selection of sex-reversed broodstock. Phenotypic observation alone is insufficient, particularly during early developmental stages or in species with homomorphic sex chromosomes. Consequently, the development of robust sex-specific DNA markers has become a critical component of modern monosex breeding programs. Such molecular tools enable precise genotypic sex identification, facilitate neomale or neofemale screening, and provide a foundation for hormone-free, marker-assisted breeding strategies, particularly in species such as snakeskin gourami, where female-biased growth offers clear production advantages.

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CHAPTER III

Transcriptome-Based SNP Discovery Revealed SNPs Associated with Sex Determination and with RNA Editing Involved in Sex Differentiation in Snakeskin Gourami (*Trichopodus pectoralis*)

3.1 Abstract

Sex-related markers in fish would provide a useful tool for both ecological and aquaculture research, particularly when information on sex chromosomes in fish remains limited. This study aimed to develop sex-related markers in the snakeskin gourami (*Trichopodus pectoralis*), which is an important freshwater fish showing female-biased growth. Transcriptome-based SNP discovery in ovaries and testes of juvenile and adult individuals was performed, and 21 candidate sex-specific RNA SNPs were identified. Using both Sanger sequencing and PACE SNP analysis in cDNA samples, 9 nucleotide variants were observed in *ift172*, *man2a1*, *crk*, *ezh2*, *cs*, *cast*, and *kap3l,* which exhibited sex-specific RNA SNPs with heterozygosity in males and homozygosity in females. In addition, *ift172*, *man2a1*, and *kap3l* demonstrated male-biased expression while *crk*, *ezh2*, and *cs* were female-biased transcripts, suggesting their involvement in gonadal differentiation and maturation. Three SNPs (SNP_19, SNP_20, and SNP_21) showed consistent DNA variants in the genome, providing useful markers for genotypic sex identification. The other 6 RNA SNPs (SNP_2, SNP_3, SNP_4, SNP_5, SNP_12, and SNP_15) were not detected in the genome. In addition, male-biased expression of *adar* was observed, which suggested that RNA editing may contribute to sex differentiation in the snakeskin gourami. A mismatch-sensitive T7EI assay validated SNP_19 and SNP_20, which provided a reliable and cost-effective approach to sex-specific DNA marker development. Overall, this study demonstrated and validated the sex-specific SNPs that could offer a useful tool for further application of sex identification in the snakeskin gourami.

Keywords: Sex determination, SNP, RNA editing, Transcriptome, Snakeskin gourami (*Trichopodus pectoralis*).

3.2 Introduction

Among vertebrates, teleost fish exhibit a wide variety of sex determination mechanisms, which may be influenced by genetic sex determination (GSD), environmental sex determination (ESD), or their interaction (Devlin & Nagahama, 2002). While several fish species are sexually dimorphic in size, body colour, or behaviour, others display no observable differences between males and females (Mesnick & Ralls, 2018; Uba, 2019; Han et al., 2020). Although the effects of ESD on sex differentiation cannot be excluded, genetic differences between males and females under GSD may serve as valuable tools in both ecological research and aquaculture investigations (Rajendiran et al., 2021). However, identification of sex chromosomes or sex-determining genes in fish remains limited (Kobayashi et al., 2013; Wang et al., 2024). As an alternative, sex-related markers have been widely investigated as practical tools in ecological and aquaculture research (Martinez et al., 2026; L. Zhang et al., 2025).

In species lacking external sexual dimorphism, molecular sex markers are especially useful for monitoring sex ratios in natural populations, thereby supporting ecological and conservation efforts (Vu et al., 2019). Temperature-dependent sex determination (TSD) may lead to male-biased populations under climate change, potentially disrupting population dynamics and threatening ecological balance and biodiversity (Geffroy & Wedekind, 2020; Ospina-Álvarez & Piferrer, 2008). Thus, sex-related markers can be used to track population-level sex ratios and assess ecological responses to thermal stress. In aquaculture, where external sexual dimorphism is often absent, sex-related markers facilitate the identification of sex, aiding artificial breeding and selective breeding programs (Abaho et al., 2025; Curzon et al., 2022). Conversely, in species that display sexual dimorphism at maturity (Kabpha et al., 2023; Mu et al., 2024), these markers are valuable for early-stage sex identification. Particularly in indirect methods of sex reversal, neomales (XX individuals phenotypically male) can be used as broodstock to produce all-female offspring (Luckenbach et al., 2017; Mu et al., 2024). In such cases, a sex-related marker is essential for identifying genotypic sex beyond relying solely on progeny sex ratios. Therefore, sex-related markers are indispensable tools in both ecological research and aquaculture, particularly where information on fish sex chromosomes remains limited.

Advances in bioinformatics and molecular genetics have facilitated the discovery of sex-specific DNA markers in the fish genome (Chen et al., 2007, 2015; Hua et al., 2023;

Vale et al., 2014; L. Zhang et al., 2025). However, other regulatory mechanisms—such as RNA editing, epigenetics, and alternative splicing—may also influence sex determination and differentiation by altering transcript-level nucleotide variants and expression levels (Lu et al., 2022; Wang et al., 2020).

Transcriptome analysis using high-throughput RNA sequencing (RNA-Seq) has emerged as a powerful method for quantifying and identifying RNA molecules in biological samples. RNA-Seq also enables identification of single-nucleotide polymorphisms (SNPs), which are valuable for discovering genetic markers (Zhang et al., 2024). Notably, transcriptome-based SNP discovery can yield putative RNA markers without requiring prior genomic sequence information. In addition, SNPs are bi-allelic, reproducible, and readily incorporated into high-throughput genotyping platforms such as PCR Allele Competitive Extension (PACE), allowing for scalable and accurate analyses (Torres et al., 2025). Furthermore, transcriptomic datasets have successfully enabled SNP marker development in species such as the black-spotted frog (*Pelophylax nigromaculatus*), black-faced blenny (*Tripterygion delaisi*), and various catfish species (Schunter et al., 2014; Wang et al., 2013; Zhang et al., 2024).

The snakeskin gourami (*Trichopodus pectoralis*) is an economically important freshwater fish, widely distributed and cultured across Southeast Asia. Females are generally larger than males; thus, feminisation using 17β-estradiol-supplemented diets has been employed to promote all-female production (Kabpha et al., 2023). Alternatively, indirect all-female production using neomales may offer a sustainable and food-safe approach for improved productivity. Despite its economic significance, the sex-determination mechanism in the snakeskin gourami remains poorly understood, and no sex-specific DNA markers have been reported to date. Previous transcriptomic analyses using RNA-Seq have identified differentially expressed genes (DEGs) between testes and ovaries, shedding light on molecular pathways involved in sex differentiation and maturation in both juvenile and adult stages (Boonanuntanasarn et al., 2020; Jantawongsri et al., 2025). However, no sex-specific molecular markers have yet been identified. In this study, we applied a transcriptome-based SNP discovery approach using juvenile and adult fish to identify and validate candidate sex-specific RNA SNPs. RT-PCR followed by Sanger sequencing and PACE SNP analysis was used for marker validation. In addition, by integrating transcriptomic and genomic data, we characterised sex-associated nucleotide variants at both RNA and DNA levels. We further demonstrated the utility of a mismatch-

sensitive T7 Endonuclease I (T7EI) assay to validate DNA-level SNP genotypes. Moreover, we provide the first evidence that RNA editing may play a role in the sex determination mechanism of the snakeskin gourami.

3.3 Materials and methods

3.3.1 Ethics statement

The study protocols for fish care and experimental procedures were reviewed and approved by the Kasetsart University Institutional Animal Care and Use Committee (ACKU 61-FIS-004) and conducted in accordance with institutional and national guidelines.

3.3.2 Experimental design, experimental fish, and sample collection

To discover sex-specific SNP markers in snakeskin gourami, RNA sequencing was performed to determine SNPs associated with sex differences in juvenile (sex differentiation stage) and adult (maturation stage). The transcriptomic resources for comparison between the testes and ovary in juvenile fish and adult fish used in this study were obtained from previously published datasets by Boonanuntanasarn et al. (2020) and Jantawongsri et al. (2025), respectively. Snakeskin gourami (*T. pectoralis*) were collected from eight commercial farms (Juvenile; 4 farms (replications), adult; 4 farms (replications) located in Samutr Sakhon, Thailand. Juvenile males (body weight, 19.22 ± 0.23 g; gonad weight, 0.01 ± 0.001 g; gonadosomatic index [GSI], 0.05 ± 0.003 %), females (body weight, 18.68 ± 0.39 g; GSI, 0.73 ± 0.03%) were used for sampling of testes (n = 50/replication, 4 replications) and ovary (n = 25/replication, 4 replications), respectively. In adult fish (harvestable size), testes (n = 10/replication, 4 replications) and ovary (n = 10/replication, 4 replications) were collected from males (body weight, 217.17 ± 9.89 g; GSI, 0.05 ± 0.005%) and females (body weight, 226.94 ± 7.69 g; GSI, 4.17 ± 0.31%). All fish were euthanized using tricaine methanesulfonate (MS-222; 150 mg/L) before dissection. Histological examination was performed to confirm gonadal sex (Boonanuntanasarn et al. 2020; Jantawongsri et al. 2025).

3.3.3 RNA sequencing, read pre-processing, *de novo* transcriptome assembly, and SNP Identification

Total RNA was isolated from approximately 100 mg of gonadal tissue (testis or ovary) using TRIzol™ reagent (Invitrogen, Carlsbad, CA, USA) and digested with

RNase-free DNase (Promega, Madison, WI, USA) according to the manufacturer's instructions. The concentration and purity of extracted RNA were evaluated using a NanoDrop™ ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and RNA integrity was assessed by 1% agarose gel electrophoresis. Further quality control was performed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) to determine the RNA integrity number (RIN). For downstream transcriptome analysis, testicular RNA samples with RIN ≥ 7.0 were selected. In contrast, ovarian RNA samples with RIN ≥ 4.5 were accepted due to the naturally high abundance of low-molecular-weight 5S rRNA observed in juvenile ovary tissues.

For transcriptome analysis in juvenile fish, four replications of testicular RNA (pooled equal amounts of RNA from fifty testes in one replication) and ovarian RNA (pooled equal amounts of RNA from twenty-five ovaries in one replication) were used for transcriptome analysis. In adult fish, four replications of testicular RNA (pooled equal amounts of RNA from ten testes) and ovarian RNA (pooled equal amounts of RNA from ten ovaries) were used. For each replicate, 2 μg of total RNA was used to construct each RNA library for juvenile ovaries (JO1, JO2, JO3, and JO4) juvenile testis (JT1, JT2, JT3, and JT4), adult ovaries (AO1, AO2, AO3, and AO4) or adult testis (AT1, AT2, AT3, and AT4) as described previously (Boonanuntanasarn et al. 2020; Jantawongsri et al. 2025). All libraries were sequenced on an Illumina HiSeq 2500 platform at the Novogene Bioinformatics Institute (Beijing, China) following the manufacturer's instructions.

Raw Illumina HiSeq 2500 sequencing data were base-called into FASTQ files and subjected to quality filtering. Clean reads were assembled *de novo* using Trinity (version 2.0.6 for juvenile and version r2014-04-13p1 for adult) with default parameters (k-mer = 25; minimum contig length = 200 bp) (Grabherr et al., 2011). Redundant transcripts were hierarchically clustered using Corset v1.05 (Davidson and Oshlack, 2014). The longest transcript from each cluster was designated as a unigene.

The *de novo* transcriptome assembly is provided as a reference for reading mapping. The clean reads were aligned back to the assembled transcriptome using Bowtie2 (Langmead and Salzberg, 2012). Read sorting and screening were performed with SAMtools and Picard, followed by SNP calling using the Genome Analysis Toolkit (GATK v3). In Juvenile fish, sex associated nucleotide variants between JO1-4 and JT1-4 were explored. In addition, nucleotide variants between AO1-4 and

AT1-4 were screened using transcriptome information in adult fish. SNPs exhibiting consistent sex-linked polymorphisms across juvenile and adult groups were identified and designated as putative sex-specific RNA SNP markers for subsequent validation.

3.3.4 Validation of candidate RNA SNPs and their corresponding DNA variants using Sanger method

3.3.4.1 Sample collection and histological study

To validate the sex-specific RNA SNP markers, gonadal samples (testes and ovaries) from 10 juvenile fish including five females (body weight = 18.4 ± 2.3 g, gonad weight = 0.15 ± 0.1 g; GSI = 0.01 ± 0.01 %) and five males (body weight = 18.6 ± 2.6 g, gonad weight = 0.01 ± 0.01 g; GSI = 0.0008 ± 0.0004 %) and 10 adult fish including five females (body weight = 225.8 ± 24.22 g, gonad weight = 17.48 ± 7.35 g; GSI = 0.08 ± 0.04 %) and five males (body weight = 132.2 ± 9.73 g, gonad weight = 0.18 ± 0.05 g; GSI = 0.001 ± 0.0003 %) were collected from farms in Samut Sakhon, Thailand. Before sampling, all fish were euthanized with 150 mg/L of MS-222, and caudal fins were also collected for genomic DNA extraction. Gonadal tissues (ovary or testis) were collected and divided into two portions: one was immediately snap-frozen in liquid nitrogen and stored at −80°C for RNA extraction, while the other was fixed in Bouin's solution at 4°C for 16–18 h. Fixed tissues were then transferred to 80 % ethanol and stored at 4°C until histological processing for sex identification.

Histological analysis was performed to confirm sex and the presence of the developmental stage of testes or ovaries. Fixed gonadal tissues were dehydrated through a graded ethanol–xylene series, embedded in paraffin, sectioned transversely at 5 µm thickness, and stained with hematoxylin and eosin (H&E) according to the protocol described previously (Kabpha et al., 2023).

3.3.4.2 Total RNA extraction, reverse transcription PCR, and sequencing

To validate SNPs identified from RNA-seq, total RNA was extracted from approximately 50-100 mg of gonadal tissue (juvenile: 5 ovaries, 5 testes; adult: 5 ovaries, 5 testes; n = 20) using the same procedure described above for RNA isolation. RNA quantity and integrity were assessed as previously outlined. Subsequently, 2 µg of RNA was reverse-transcribed into first-strand cDNA using the SuperScript™ III Reverse Transcriptase Kit (Invitrogen) with random hexamer (Promega), following the manufacturer's instructions.

According to screening sex-specific SNPs in juvenile and adult fish, a total of 21 putative sex-specific SNP candidates consistently identified across juvenile and adult gonadal transcriptomes were selected for validation (Table 3.1). Primer pairs were designed using Primer3Plus (Table 3.2). Reverse transcription PCR (RT-PCR) amplification was performed using TaKaRa Ex Taq polymerase (TaKaRa Bio, Shiga, Japan) in a total reaction volume of 25 µL. Each reaction mixture contained 2.5 µL of 10× Ex Taq Buffer, 2 µL of dNTP Mixture (2.5 mM each), 0.125 µL of TaKaRa Ex Taq polymerase (5 U/µL), 2.5 µL of forward primer (2 µM), 2.5 µL of reverse primer (2 µM), 2.5 µL of 20× diluted cDNA template, and 12.875 µL of nuclease-free water. RT-PCR cycling was conducted under the following conditions: 95°C for 3 min; 35 cycles of 95°C for 30 s, annealing at the optimal temperature for each primer pair (55–65°C) for 30 s, and extension at 72°C for 25 s; followed by a final extension at 72°C for 10 min. The target PCR products were excised from agarose gels and purified using the QIAEX® II Gel Extraction Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol for Sanger sequencing. Only SNPs in which females consistently exhibited homozygous genotypes and males heterozygous genotypes in cDNA with transcriptome-based SNPs were retained for further validation using the PACE SNP genotyping assay. In addition, their functional annotation of the assembled transcriptome and the differentially expressed levels, presented as $\log_2$ fold change (male/female) values, was conducted following the procedures described in Boonanuntanasarn et al. (2020) and Jantawongsri et al. (2025).

Table 3.1 Sex-associated transcriptome-based SNPs in juvenile and adult snakeskin gourami.

SNPs	Clusters	Location in mRNA	Genotypes Female	Male
SNP_1	Cluster-22178.58277	3′-UTR	A/A	A/G
SNP_2	Cluster-22178.80501	3′ UTR	A/A	A/G
SNP_3	Cluster-22178.68092	3′ UTR	A/A	A/G
SNP_4	Cluster-22178.66311	3′ UTR	A/A	A/G
SNP_5	Cluster-22178.77480	CDS	A/A	A/G
SNP_6	Cluster-22178.67537	3′ UTR	A/A	A/G
SNP_7	Cluster-22178.36261	3′ UTR	A/A	A/G
SNP_8	Cluster-22178.64482	CDS	A/A	A/G
SNP_9	Cluster-22178.72264	3′ UTR	A/A	A/G

Table 3.1 (Cont.)

SNPs	Clusters	Location in mRNA	Genotypes Female	Male
SNP_10	Cluster-22178.76218	3′ UTR	C/C	C/A
SNP_11	Cluster-22178.86555	3′ UTR	A/A	A/G
SNP_12	Cluster-22178.67781	3′ UTR	A/A	A/G
SNP_13	Cluster-22178.82477	3′ UTR	A/A	A/G
SNP_14	Cluster-22178.67640	CDS	A/A	A/G
SNP_15	Cluster-22178.56828	3′ UTR	A/A	A/G
SNP_16	Cluster-22178.90734	CDS	A/A	A/G
SNP_17	Cluster-22178.75619	5′ UTR	T/T	T/A
SNP_18	Cluster-22178.91475	3′ UTR	G/G	G/C
SNP_19	Cluster-22178.57271	3′ UTR	G/G	G/T
SNP_20	Cluster-22178.57271	3′ UTR	C/C	C/T
SNP_21	Cluster-22178.57271	CDS	G/G	G/A

See nucleotide sequences in Appendix.

3.3.4.3 Genomic DNA extraction, DNA variant analysis using Sanger sequencing

Genomic DNA (gDNA) was extracted from approximately 100 mg of fin tissue collected from juvenile (5 females and 5 males) and adult (5 females and 5 males) snakeskin gourami using the standard phenol–chloroform extraction method. DNA concentration and purity were measured using a NanoDrop® spectrophotometer, and DNA integrity was verified by electrophoresis on a 0.7% agarose gel.

Candidate putative sex-specific RNA SNPs that were consistently detected in both juvenile and adult transcriptomes were selected for genomic validation. Since SNP_21 contained an intron part, another pair of primers was designed for Sanger sequencing and PACE SNPs genotyping assay (Table 3.2). A total of 20 individuals, identical to the cDNA validation sample (juveniles: 5 females and 5 males; adults: 5 females and 5 males), were tested using PCR. The PCR condition was performed as described above for cDNA validation. Subsequently, the target PCR products were excised from agarose gels and purified using the QIAEX® II Gel Extraction Kit (Qiagen) following the manufacturer's protocol for Sanger sequencing.

Table 3.2 Primers used for validating sex-specific SNP markers.

Name	Clusters	Primers (5′-3′)	Tm (°C)	Products (bp)	Application
SNP_1	Cluster-22178.58277	F: AATACGACTCACTATAGGGCGTGAAGGAACACGCGGAG R: TCAGCACATTTCCTGCCAGCTACA	65	517	Sanger sequencing
SNP_2	Cluster-22178.80501	F: CAGGCTTAGTGGTAGCTACACA R: GCATTGGTCGTTCAGTCCGA	60	308	Sanger sequencing
SNP_3	Cluster-22178.68092	F: CCGCATAGAGATCGACTGAAT R: TAAGTGTGGCCTTCCTGGTG	58	307	Sanger sequencing
SNP_4	Cluster-22178.66311	F: CCGGGTGAGGTTGTCAGTTT R: TCACAGTGCTCAGCTCTCAC	60	300	Sanger sequencing
SNP_5	Cluster-22178.77480	F: CAGCCCTGTGACTCCTCTTG R: AACCAGCCACGTCTGAAGG	60	289	Sanger sequencing
SNP_6	Cluster-22178.67537	F: AATACGACTCACTATAGGGGGGTTTGGCTAACTTTGCA R: GTTCTGCAACACATGGCAAC	60	619	Sanger sequencing
SNP_7	Cluster-22178.36261	F: AATACGACTCACTATAGGGACCTGATCTGTTCAGTGGCCTCCC R: GTGCACCGAGTGAGCCTCCG	65	665	Sanger sequencing
SNP_8	Cluster-22178.64482	F: AATACGACTCACTATAGGGACGCCCTCGCTTTCCCCC R: GTCTGAGAAATCGACAGTCTGCTGGC	65	236	Sanger sequencing
SNP_9	Cluster-22178.72264	F: GGGAGTTTAAGAAACATATGGAAGCG R: CATGCTGCTGCTCCCCAG	60	289	Sanger sequencing

Table 3.2 (Cont.).

Name	Clusters	Primers (5′-3′)	Tm (°C)	Products (bp)	Application
SNP_10	Cluster-22178.76218	F: AATACGACTCACTATAGGGCAGAAGTCAAAGGTACCATACAGGCT R: TGTCAACATGTGTTACTGGTCCCAGAC	65	519	Sanger sequencing
SNP_11	Cluster-22178.86555	F: AATACGACTCACTATAGGGAGTCTTGAACTGTCACGCATGCTC R: CAGGTATGAGAGTGGTGTCTAGTCAGT	65	518	Sanger sequencing
SNP_12	Cluster-22178.67781	F: ACTCTATGAACACTCCACTTTAAAAGT R: TGTACATTACTGTTGCTGTACTTCA	58	315	Sanger sequencing
SNP_13	Cluster-22178.82477	F: TCCTGAATGCCTTTGTGTTTTT R: ACTGGCATCATGTGTGACCT	58	317	Sanger sequencing
SNP_14	Cluster-22178.67640	F: CAGCCTCCAGTTTCCTGTCC R: GGAGTAAACCCGTTCCGACC	60	319	Sanger sequencing
SNP_15	Cluster-22178.56828	F: CTGCTCACCTCCTTGCATGT R: TGTAAACCACGTGCTGTCCA	60	313	Sanger sequencing
SNP_16	Cluster-22178.90734	F: TGAAGAGGAAGCATCAGGATCC R: TTTTTCCCAAGGTTGCCACC	59	200	Sanger sequencing
SNP_17	Cluster-22178.75619	F: AATACGACTCACTATAGGGTGCCAGGCTCCCCTGTCTCC R: AAGCTTCCAGGAAACCGACAGTGA	65	518	Sanger sequencing
SNP_18	Cluster-22178.91475	F: AGCTGGCGGCAGATTTCTAA R: ACACCAGAAACACCTCAGGA	60	322	Sanger sequencing

Table 3.2 (Cont).

Name	Clusters	Primers (5′-3′)	Tm (°C)	Products (bp)	Application
SNP_19, 20	Cluster-22178.57271	F: AATACGACTCACTATAGGGCCAGGGCTGTAACTCTGGACT R: GCACCTTGATCAAAGCACTCTCCA	63	551	Sanger sequencing
SNP_19, 20	Cluster-22178.57271	F: AATACGACTCACTATAGGGCCAGGGCTGTAACTCTGGACT R: GCACCTTGATCAAAGCACTCTCCA	63	551	Sanger sequencing
SNP_21 (cDNA)	Cluster-22178.57271	F:AATACGACTCACTATAGTCTACAACTTGCCCGCAACC R:CCACCAGAACCAGAAGCTCC	60	520	Sanger sequencing
SNP_21 (gDNA)	Cluster-22178.57271	F: GAGACAGCTCTAGGGGCTCT R: TGCTCCTGTTTGGCCAGAAG	60	518	Sanger sequencing
SNP_2 (PACE)	Cluster-22178.80501	CRP:CTGTTGATGGATCTGCTATAGAACTTCAT ASP-A:GAAGGTGACCAAGTTCATGCTGTAATTATTATTACTGTCATCAAATCC**T** ASP-G:GAAGGTCGGAGTCAACGGATTCTGTAATTATTATTACTGTCATCAAATCC**C**	60	109 (ASP-A) 111 (ASP-G)	PACE genotyping
SNP_3 (PACE)	Cluster-22178.68092	CRP:GAAAGACATCCAGACACCATGACTGAA ASP-A:GAAGGTGACCAAGTTCATGCTCATCCACATGAGGTGATCCAGA**A** ASP-G:GAAGGTCGGAGTCAACGGATTCATCCACATGAGGTGATCCAGA**G**	60	73	PACE genotyping
SNP_4 (PACE)	Cluster-22178.66311	CRP:AAGGAACAGACTGACATTCTGGAAAAGAT ASP-A:GAAGGTGACCAAGTTCATGCTATTGTACTTATCTCACTCAAGAGCAAAT**A** ASP-G:GAAGGTCGGAGTCAACGGATTGTACTTATCTCACTCAAGAGCAAAT**G**	60	95 (ASP-A) 92 (ASP-G)	PACE genotyping

Table 3.2 (Cont).

Name	Clusters	Primers (5′-3′)	Tm (°C)	Products (bp)	Application
SNP_5 (PACE)	Cluster-22178.77480	CRP:CACACTCCCTCACTGCCAGGTA ASP-A:GAAGGTGACCAAGTTCATGCTGTTGCAAAGCCCAGTGTAACACT**A** ASP-G:GAAGGTCGGAGTCAACGGATTGCAAAGCCCAGTGTAACACT**G**	60	81 (ASP-A) 78 (ASP-G)	PACE genotyping
SNP_12 (PACE)	Cluster-22178.67781	CRP:ACAACTTTGAAAAAAGGGAGACCAAAGATT ASP-A:GAAGGTGACCAAGTTCATGCTCCATAGTTAAAAGATACTGCCCCTTT**A** ASP-G:GAAGGTCGGAGTCAACGGATTCATAGTTAAAAGATACTGCCCCTTT**G**	60	78 (ASP-A) 77 (ASP-G)	PACE genotyping
SNP_15 (PACE)	Cluster-22178.56828	CRP:CAGAGATCATCTCAGCTGGCAGTTT ASP-A:GAAGGTGACCAAGTTCATGCTGCCATCTTTCCAACAAACCTCAC**T** ASP-G:GAAGGTCGGAGTCAACGGATTCCATCTTTCCAACAAACCTCAC**C**	60	70	PACE genotyping
SNP_19, 20 (PACE)	Cluster-22178.57271	CRP:CTACTAATGCATTAAATGTCCTAAGCTCCC ASP-GC:GAAGGTGACCAAGTTCATGCTAATATTGAGCATTTACAATACTGGACAT**GC** ASP-TT:GAAGGTCGGAGTCAACGGATTAATATTGAGCATTTACAATACTGGACAT**TT**	60	100	PACE genotyping
SNP_21 (cDNA, (PACE)	Cluster-22178.57271	CRP:CACCTATTTTATAATGACTCACCACTCCAT ASP-G:GAAGGTGACCAAGTTCATGCTTTCTGCTTCTCTAGTTTCTCCCA**G** ASP-A:GAAGGTCGGAGTCAACGGATTTTTCTGCTTCTCTAGTTTCTCCCA**A**	60	55	PACE genotyping

Table 3.2 (Cont).

Name	Clusters	Primers (5′-3′)	Tm (°C)	Products (bp)	Application
SNP_21 (gDNA, (PACE)	Cluster-22178.57271	CRP:ATAGTGTGGAGCTGGCCACCATTAT ASP-G:GAAGGTGACCAAGTTCATGCTTATAATGACTCACCACTCCATGAAA**C** ASP-A:GAAGGTCGGAGTCAACGGATTTTATAATGACTCACCACTCCATGAAA**T**	60	195 (ASP-G) 196 (ASP-A)	PACE genotyping

Abbreviation: Tm (°C) =Annealing temperature (°C); bp= base pair; ASP=Allele-specific forward primers; CRP=Common reverse primer; PACE=PCR Allele Competitive Extension.

Note: The underlined sequence (AATACGACTCACTATAGGG) corresponds to the T7 universal sequencing tag, which was added at the 5′ end of primers to facilitate sequencing. Underline indicates the universal FAM (GAAGGTGACCAAGTTCATGCT) and HEX (GAAGGTCGGAGTCAACGGATT) tails added to the 5′ end of allele-specific primers.

3.3.5 Validation of candidate RNA SNPs and their corresponding DNA variants using PACE SNP analysis

To strengthen the validation of sex-specific RNA SNP markers across a broader population, an additional cohort of 20 fish was collected from farms in Samut Sakorn and the Suranaree University of Technology Farm (SUT Farm), Thailand. The sample included 10 juveniles (5 females and 5 males) and 10 adults (5 females and 5 males); detailed morphometric and gonadosomatic index (GSI) data are summarized in Table 3.3. All fish were euthanized with an overdose of MS-222 (150 mg/L) before tissue collection. Caudal fin clips were collected from each individual and preserved for gDNA extraction. Gonadal tissues (ovaries or testes) were dissected, immediately snap-frozen in liquid nitrogen, and stored at -80°C until RNA extraction. Total DNA and RNA were extracted from the fin and gonad tissues, respectively, following previously described methods. Subsequently, cDNA was synthesized from the RNA extracts. For the PACE SNP analysis, allele-specific primers were designed using the PACE® Assay Design Template (3CR Bioscience Ltd., UK). Each assay consisted of two allele-specific forward primers, each carrying a universal FAM or HEX fluorescent tail, and one common reverse primer. Each assay consisted of two allele-specific forward primers (ASPs) carrying universal FAM or HEX fluorescent tails and one common reverse primer (CRP) (Table 3.2). SNP genotyping was conducted using the PACE® 2.0 Genotyping Master Mix (3CR Bioscience, Harlow, UK) following the manufacturer's instructions. The reaction (a total volume of 10 µL) consisted of 5 µL of 2× PACE 2.0 Genotyping Master Mix, 0.138 µL of custom-designed Assay Mix (containing two allele-specific forward primers (12 µM) and one common reverse primer (30 µM)), and 25 ng of cDNA template. PCR amplification was performed under the following cycling conditions: initial enzyme activation at 94°C for 15 min; followed by 10 cycles of 94°C for 20 s and 65–57°C for 60 s (decreasing 0.8°C per cycle); and then 30 cycles of 94°C for 20 s and 57°C for 60 s. After amplification, the fluorescence signals of FAM and HEX were recorded at or below 40°C in endpoint mode. The genotypes were determined based on the relative FAM and HEX fluorescence intensities, and cluster plots were generated for allele discrimination. The no-template control (NTC) wells showed no detectable fluorescence in either channel, confirming the absence of cross-contamination.

To further verify the sex-specific SNPs at the genomic level, the PACE SNP genotyping assay was performed using the same gDNA samples from 20 individuals. The PACE® reaction setup, primer composition, PCR cycling conditions, fluorescence signal detection, and genotype determination followed the procedures described for cDNA validation. Genotypic profiles were then compared between male and female fish to evaluate whether the SNPs identified in cDNA were also present at the genomic level.

Table 3.3 Morphometric and gonadal characteristics of the independent validation cohort.

Group	Sex	Body Weight (g)	Gonad Weight (g)	GSI (%)
Juvenile	Female (n=5)	14.4 ± 4.62	0.12 ± 0.03	0.01 ± 0.002
	Male (n=5)	18.6 ± 2.61	0.01 ± 0.01	0.0008 ± 0.0004
Adult	Female (n=5)	223.2 ± 25.09	18.67 ± 7.24	0.08 ± 0.04
	Male (n=5)	128.4 ± 27.19	0.19 ± 0.08	0.001 ± 0.0004

Note: Values are presented as mean ± SD (n = 5). GSI (gonadosomatic index) = (gonad weight/body weight) × 100. Juvenile females showed slightly higher GSI values than males, while adult females exhibited markedly higher gonad weight and GSI compared with males.

3.3.6 Validation of sex specific DNA SNPs in other fish populations

3.3.6.1 Sample collection and DNA variant analysis using Sanger sequencing

This study validated the sex-specific SNP markers (SNP_19, SNP_20, and SNP_21) in four fish populations (SUT1, SUT2, SUT3, and SUT4) that were obtained from the SUT farm. Adult fish that demonstrated obvious sexual dimorphism in body shape were anesthetized with 150 mg/L of MS-222. Caudal fin samples were collected from 100 adult fish, including fifty females (body weight: 211.84 ± 25.78 g; gonad weight: 18.43 ± 4.96 g; GSI: 0.09 ± 0.03 %) and fifty males (body weight: 124.24 ± 2.6 g; gonad weight: 0.17 ± 0.05 g; GSI: 0.001 ± 0.0003 %). Genomic DNA was extracted using fin tissues (100 mg) following the same procedure described above for gDNA

isolation. All 100 individuals (50 females and 50 males) were genotyped by PCR amplification and Sanger sequencing to confirm the SNP genotypes.

3.3.6.2 DNA variant genotyping using PACE SNP assay

To further validate the sex-specific SNP markers (SNP_19, SNP_20, and SNP_21) at the population level, genotyping was conducted using the PACE SNP assay. gDNA from 100 individuals (50 females and 50 males) previously analyzed by Sanger sequencing was used as templates. The PACE® reaction setup, primer composition, PCR cycling conditions, fluorescence detection, and genotype calling were performed following the same protocol used for cDNA validation. The NTC wells were included in each run to monitor potential contamination.

3.3.6.3 DNA variant genotyping using the T7 Endonuclease I assay

A PCR-based T7EI digestion for RNA SNP_19, SNP_20, and SNP_21 and their DNA variants was conducted in both male (n =10) and female (n =10). Purified PCR products (400 ng) from cDNA and gDNA (the same PCR products for sequencing) were used for analysis of SNP-induced heteroduplex formation using T7 Endonuclease I (New England Biolabs, USA) according to the manufacturer's instructions. The mixture was denatured at 95°C for 5 min and annealed using a two-step cooling protocol: from 95°C to 85°C at 2°C/s, then from 85°C to 25°C at 0.1°C/s, before being held at 4°C. Subsequently, 1 µL of T7EI enzyme was added to the reaction mixture (final volume = 20 µL) and incubated at 37°C for 15 min. The reaction was terminated by adding 1.5 µL of 0.25 M EDTA. Digested products were resolved on 1.5 % agarose gels by electrophoresis.

3.4 Results

3.4.1 Sex-specific SNPs identified from gonadal transcriptomes of juvenile and adult *T. pectoralis*

This study used transcriptome information of *T. pectoralis* comparing testes and ovaries between sexes in juvenile (JT1–4; 179,323,982 clean reads vs JO1–4; 190,792,984 clean reads) and adult stages (AT1–4; 174,826,234 clean reads vs AO1–4; 179,753,200 clean reads) (Table 3). *De novo* assembly and annotation yielded 50,517 unigenes for juveniles and 55,883 unigenes for adults, which were used as references for SNP discovery (Tabe 4). In total, 65,535 polymorphic SNPs associated with sex

differences were identified across 380 individuals (JO1-4: 100 fish, JT1-4: 200 fish, AO1-4: 40 fish, AT1-4: 40 fish) (Table S1; see Appendix). Comparative transcriptomic analysis revealed 24 putative sex-specific SNPs between JO1-4 and JT1-4 (Table S2; see Appendix) and 389 SNPs between AO1-4 and AT1-4 (Table S3; see Appendix). By integrating SNP information from both juvenile and adult stages, 21 candidate sex-specific RNA SNPs were obtained in *T. pectoralis*. These SNPs were distributed across different transcript clusters, and SNP positions were located within transcribed regions, including 5'-untranslated region (5'-UTR), coding DNA sequence (CDS), and 3'-untranslated region (3'-UTR) (Table 3.1).

3.4.2 Validation of candidate sex-specific RNA SNPs

This study validated the candidate sex-specific RNA SNPs using testicular and ovarian cDNA from juvenile (5 males and 5 females) and adult fish (5 males and 5 females), and a histological study was conducted to confirm testicular and ovarian tissues. Figure 3.1 shows the representative of immature ovary and testis in juvenile and mature ovary and testis in adult fish. In juveniles, ovaries contained oogonia (OG) and previtellogenic oocytes (PVO) (Figure 3.1A and 3.1B). In adults, ovaries exhibited a full spectrum of oocyte development, and there are various developmental stages of oocytes, including OG, PVO, early vitellogenic oocytes (EVO), and mature oocytes (MO) (Figure 3.1C and 3.1D). Juvenile testes displayed the typical lobular testes containing spermatogonia (SG), primary spermatocytes (PSC), secondary spermatocytes (SSC), and a low proportion of spermatid (ST) (Figure 3.1E and 3.1F). In adults, testes exhibited developed spermatogenesis, with lobules characterized by the presence of SG, PSC, SSC, and a high proportion of ST (Figure 3.1G and 3.1H). These ovaries and testes were used to validate the candidate sex-specific RNA SNPs.

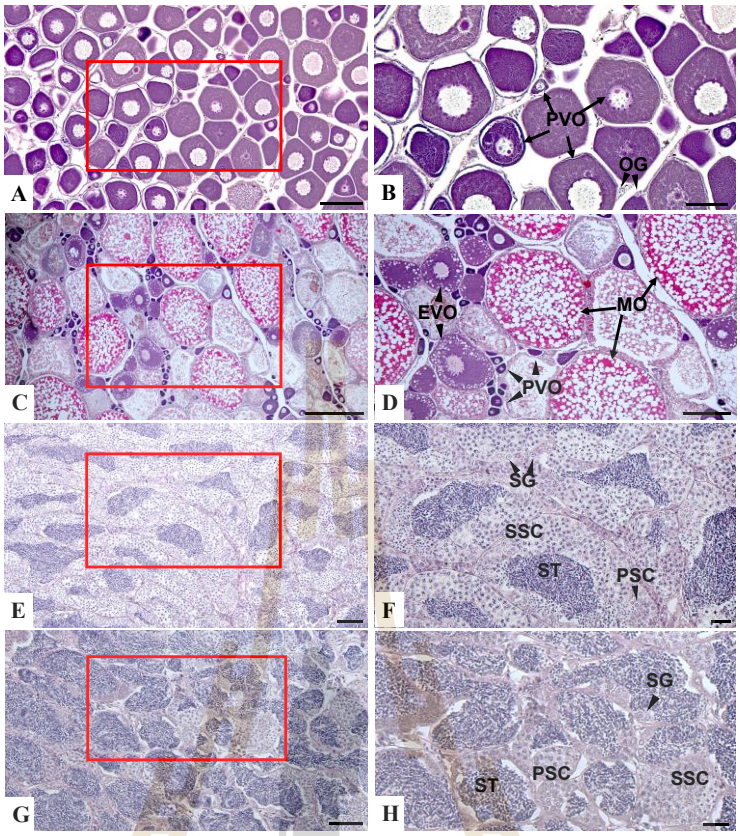

Figure 3.1 Histological characterization of juvenile and adult gonads in snakeskin gourami. The left shows representative histological sections of juvenile ovary (A), adult ovary (C), juvenile testis (E), and adult testis (G). Panels of right (B, D, F, H) represent higher magnification views of the boxed regions in A, C, E, G, respectively. Ovarian cell types observed include oogonia (OG), previtellogenic oocyte (PVO), early vitellogenic oocytes (EVO), and maturing oocyte (MO). Testicular cell types include spermatogonia (SG), primary spermatocytes (PSC), secondary spermatocytes (SSC), and spermatids (ST). Scale bars: 100 μm (A, C), 50 μm (B, D, E, G), and 20 μm (F, H).

The RT-PCR followed by Sanger sequencing showed that among 21 candidate sex-specific RNA SNPs, RNA variants of nine SNPs, including SNP_2, SNP_3, SNP_4, SNP_5, SNP_12, and SNP_15, SNP_19, SNP_20, and SNP_21, exhibited concordant genotypes with those of transcriptome-based SNPs (Table 6; Figure 3.2). Males were heterozygous A/G, A/G, A/G, A/G, A/G, A/G, G/T, C/T, and G/A, respectively, whereas female samples presented homozygous A/A, A/A, A/A, A/A, A/A, A/A, G/G, C/C, and G/G, respectively

(Figure 3.2). Using the PACE assay, nine candidate SNPs (SNP_2, SNP_3, SNP_4, SNP_5, SNP_12, SNP_15, SNP_19, SNP_20, and SNP_21) exhibited genotype patterns consistent with sex, showing predominantly homozygosity in females and heterozygosity in males (Figure 3.3). Distinct fluorescence clusters were clearly separated for most loci (e.g., SNP_2, SNP_3, SNP_4, SNP_19, and SNP_20), while partial overlaps between male and female genotypes were observed at SNP_5, SNP_12, and SNP_15. These results were overall in agreement with those obtained from RT-PCR followed by Sanger sequencing.

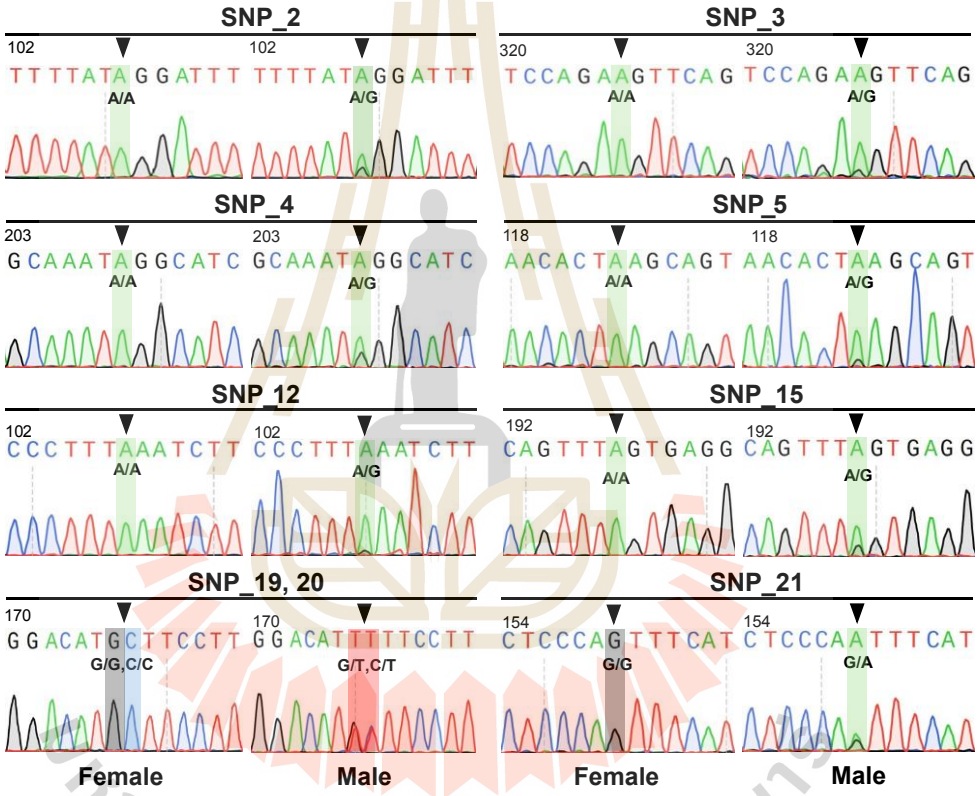

Figure 3.2 Validation of sex-associated SNPs at the RNA level by Sanger sequencing in snakeskin gourami. Representative chromatograms from RT-PCR amplified cDNA show sex-specific genotypes of SNP_2, SNP_3, SNP_4, SNP_5, SNP_12, and SNP_15 in which females consistently exhibited homozygous A/A genotypes, whereas males were heterozygous A/G. For SNP_19, SNP_20, and SNP_21, male chromatograms displayed overlapping peaks corresponding to heterozygous genotypes (e.g., G/T, C/T, or G/A), while female chromatograms showed uniform homozygous bases (e.g., G/G or C/C).

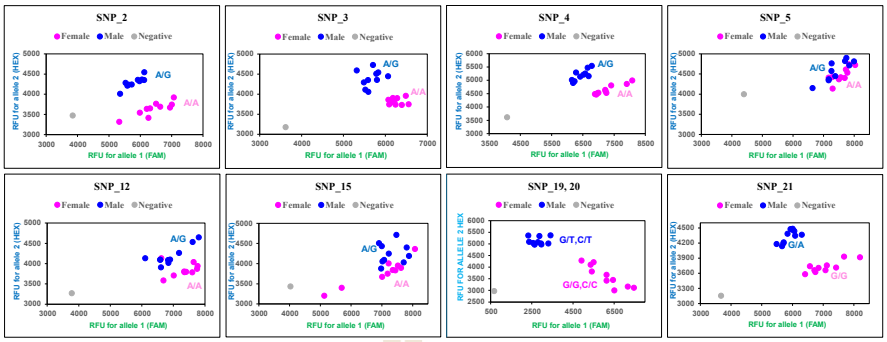

Figure 3.3 PACE genotyping analysis of nine SNPs using cDNA in snakeskin gourami. Scatter plots show fluorescence signal intensities (RFU) for allele 1 (FAM, x-axis) and allele 2 (HEX, y-axis). Each point represents an individual fish (pink = female; blue = male; gray = negative control). SNP_2, SNP_3, SNP_4, SNP_5, SNP_12, and SNP_15 exhibited female homozygosity (A/A) and male heterozygosity (A/G), whereas SNP_19 and SNP_20 showed homozygous G/G, C/C (female) and heterozygous G/T, C/T (male) genotypes, and SNP_21 displayed homozygous G/G (female) and heterozygous G/A (male) genotypes.

3.4.3 Differential expression and transcript annotation of 9 sex-associated SNPs in juvenile and adult gonads of *T. pectoralis*

Transcript annotation revealed that the nine validated SNPs (SNP_2, SNP_3, SNP_4, SNP_5, SNP_12, SNP_15, SNP_19, SNP_20, and SNP_21) were distributed across seven genes: *ift172*, *man2a1*, *crk*, *ezh2*, *cs*, *cast*, and *kap3l* (Table 3.4). Among these, SNP_5 and SNP_21 were located in CDS, whereas the others were situated in 3′UTRs (Table 3.4; Figure S1; see Appendix). Specifically, SNP_5 in *ezh2* resulted in a non-synonymous substitution (AAG⟶GAG; K⟶E), while SNP_21 in *kap3* lwas synonymous. The remaining 3′UTR SNPs—SNP_2 (*ift172*), SNP_3 (*man2a1*), SNP_4 (*crk*), SNP_12 (*cs*), SNP_15 (*cast*), SNP_19, and SNP_20 (*kap3l*) (Table 3.4).

Differential expression analysis revealed distinct sex-biased transcriptional patterns among six genes (Table 3.4). For instance, *ift172* (both juvenile and adult), *man2a1* (both juvenile and adult), and *kap3l* (adult only) exhibited male-biased expression. In contrast, *crk* (both juvenile and adult), *cs* (adult only), and *ezh2* (adult only) showed significantly higher expression in females than in males. Note that the *cast* showed no significantly different expression level (Table 3.4).

Table 3.4 Validated sex-associated SNPs and their differential expression in gonadal tissues of juvenile and adult snakeskin gourami.

SNPs	Gene name	Gene description	Genotypes		Juvenile			Adult		
			Female	Male	Sex presenting upregulation	Log2 fold change	q-value	Sex presenting upregulation	Log2 fold change	q-value
SNP_2	*ift172*	Intraflagellar transport protein 172	A/A	A/G	Male	4.323	<0.001	Male	3.829	<0.001
SNP_3	*man2a1*	mannosidase, alpha, class 2A, member 1	A/A	A/G	Male	1.024	0.035	Male	2.096	<0.001
SNP_4	*crk*	v-crk avian sarcoma virus CT10 oncogene	A/A	A/G	Female	1.158	<0.001	Female	0.953	<0.001
SNP_5	*ezh2*	enhancer of zeste 2 polycomb repressive complex 2 subunit	A/A	A/G	ns	-	-	Female	0.676	0.039
SNP_12	*cs*	citrate synthase	A/A	A/G	ns	-	-	Female	1.193	<0.001
SNP_15	*cast*	calpastatin	A/A	A/G	ns	-	-	ns	-	-
SNP_19			G/G	G/T	ns	-	-	Male	1.191	<0.001
SNP_20	*kap3l*	kinesin-associated protein 3	C/C	C/T	ns	-	-	Male	1.191	<0.001
SNP_21			G/G	G/A	ns	-	-	Male	1.191	<0.001

ns = non-significance.

See nucleotide sequence in Appendix.

3.4.4 Validation of candidate sex-specific DNA SNPs in the genome using Sanger sequencing, PACE SNP analysis

Following cDNA validation, genotyping of gDNA from 10 females and 10 males by PCR and Sanger sequencing demonstrated, among 9 sex-specific RNA SNPs, three SNPs, SNP_19 (G/T), SNP_20 (C/T), and SNP_21 (G/A) showed consistent sex-specific genotypes at the genomic level: all male individuals were heterozygous, whereas all female individuals were homozygous (Figure 3.4). Six SNPs (SNP_2, SNP_3, SNP_4, SNP_5, SNP_12, and SNP_15) exhibited no sex-specific SNPs in the genome, as all individuals were homozygous (A/A) (Figure 3.4). Consistently, PACE assays using gDNA also showed that SNP_19, SNP_20, and SNP_21 displayed male heterozygosity and female homozygosity, while SNP_2, SNP_3, SNP_4, SNP_5, SNP_12, and SNP_15 were homozygous in both sexes (Figure 3.5).

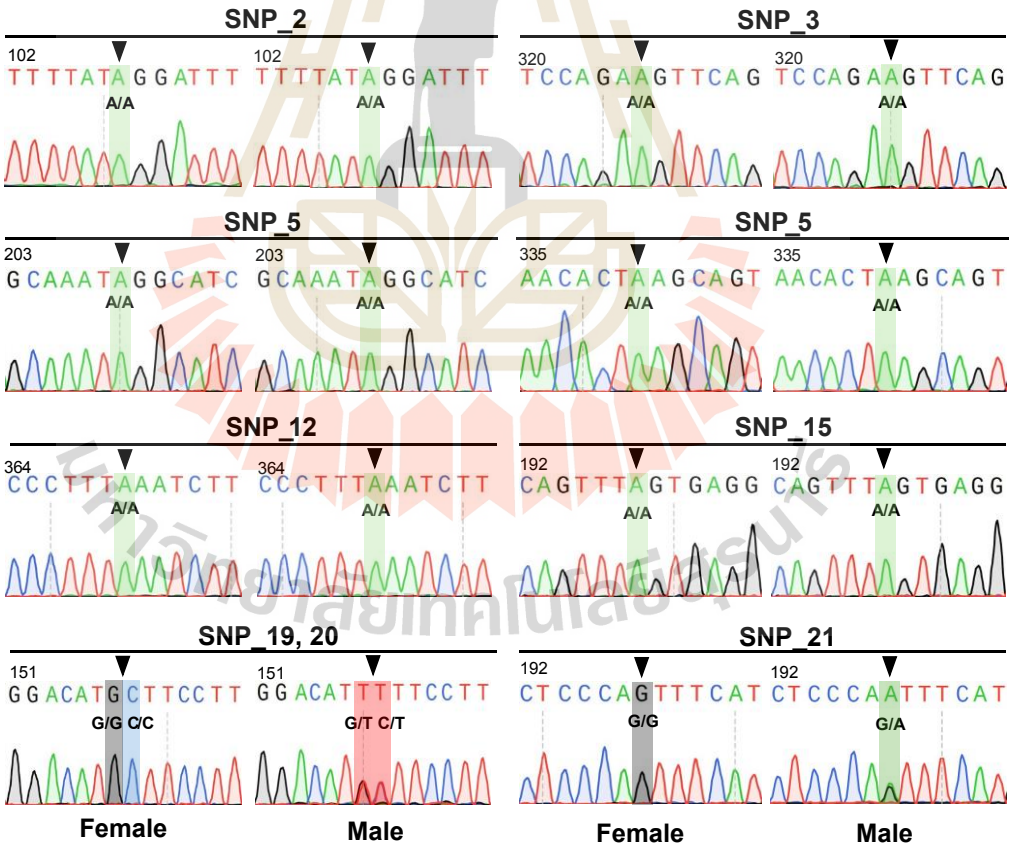

Figure 3.4 Validation of sex-specific SNPs in gDNA in snakeskin gourami using Sanger sequencing. Representative chromatograms are shown for female (left) and

male (right) individuals. SNP_2, SNP_3, SNP_4, SNP_5, SNP_12, and SNP_15 were consistently homozygous (A/A) in female and male genomes. In contrast, males exhibited heterozygosity at SNP_19 (G/T), SNP_20 (C/T), and SNP_21 (G/A), whereas females exhibited homozygosity at SNP_19 (G/G), SNP_20 (C/C), and SNP_21 (G/G).

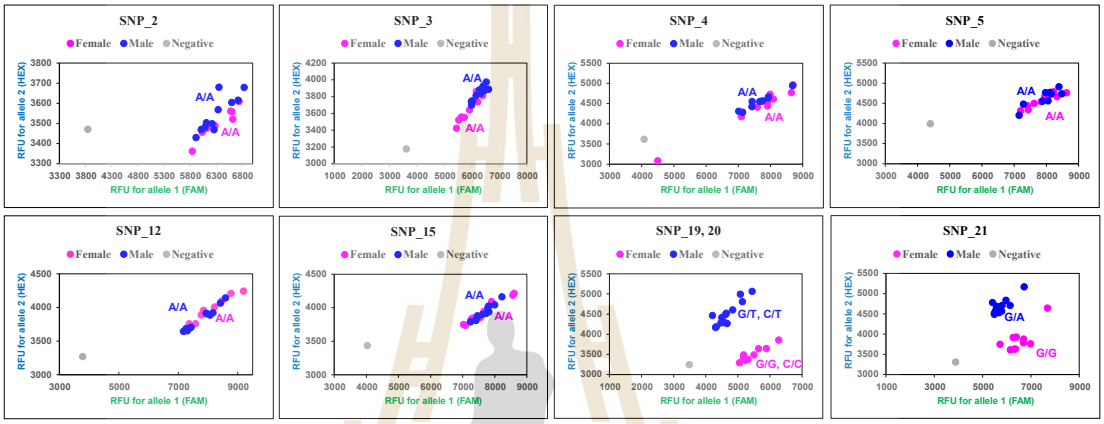

Figure 3.5 PACE genotyping of nine SNPs using gDNA from snakeskin gourami. Scatter plots show fluorescence signal intensities (RFU) for allele 1 (FAM, x-axis) and allele 2 (HEX, y-axis). Each point represents an individual fish (pink = female; blue = male; gray = negative control). For SNP_2, SNP_3, SNP_4, SNP_5, SNP_12, and SNP_15, both sexes clustered as A/A homozygotes. In contrast, SNP_19 and SNP_20 segregated by sex (females G/G, C/C, males G/T, C/T), and SNP_21 showed females G/G and males G/A.

3.4.5 Differential expression in *adar* might be associated with RNA editing in males

Six RNA SNPs (SNP_2, SNP_3, SNP_4, SNP_5, SNP_12, and SNP_15) showed no sex-specific variation in gDNA, as all individuals were homozygous (A/A), while their mRNA sequences displayed heterozygous (A/G). This incongruence between transcriptomic and genomic genotypes indicates post-transcriptional RNA editing. Supporting this interpretation, our RNA-seq analysis showed that *adar* encoding the adenosine deaminase acting on RNA was significantly upregulated in males compared to females at both developmental stages (Figure 3.6). Specifically, *adar* expression was

4.67-fold higher in juvenile males ($\log_2$ fold change = 4.6716, q = 8.19 × 10^{-71}) and 3.79-fold higher in adult males ($\log_2$ fold change = 3.7861, q = 1.59 × 10^{-64}).

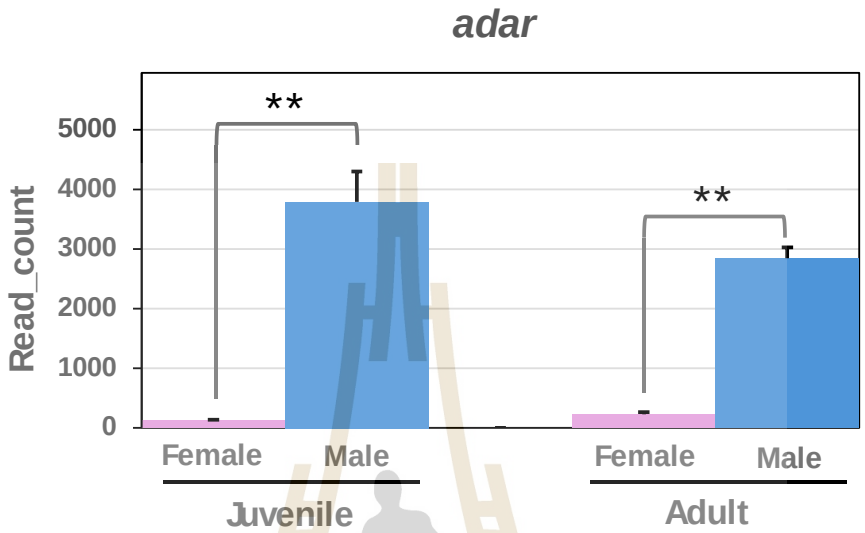

Figure 3.6 Expression levels of *adar* in juvenile and adult snakeskin gourami. Bar plots represent normalized read counts (mean ± SD) for males and females. In juveniles, *adar* expression was significantly higher in males than in females ($\log_2$ fold change = 4.6716, q-value = 8.19 × 10^{-71}). In adults, *adar* also exhibited male-biased expression ($\log_2$ fold change = 3.7861, q-value = 1.59 × 10^{-64}). Asterisks indicate statistically significant differences between sexes (P < 0.01).

3.4.6 Validation of sex specific SNPs in SNP_19, SNP_20, and SNP_21 in other fish populations by Sanger sequencing and PACE assay

The sex-specific SNP markers SNP_19, SNP_20, and SNP_21 were further validated in four fish populations (SUT1–SUT4) from the SUT Farm using both Sanger sequencing and the PACE SNP genotyping assay. Genotyping of gDNA from 50 females and 50 males consistently revealed sex-linked patterns across all populations. In both assays, all males exhibited heterozygous genotypes, SNP_19 (G/T), SNP_20 (C/T), and SNP_21 (G/A), whereas all females were homozygous for SNP_19 (G/G), SNP_20 (C/C), and SNP_21 (G/G) (Figure 3.4; Table 3.5).

Table 3.5 Validation of sex-specific SNPs in different fish populations.

Population	No. of fish	SNP_19 and SNP_20		SNP_21	
		Female (GC/GC)	Male (GC/TT)	Female (G/G)	Male (G/A)
SUT1	4	2	2	2	2
SUT2	20	10	10	10	10
SUT3	60	30	30	30	30
SUT4	16	8	8	8	8

See nucleotide sequences in Appendix.

3.4.7 Identification of RNA SNPs of SNP_19, SNP_20, and SNP_21 and their DNA variants using T7EI

This study performed a PCR-based T7EI digestion for RNA SNP_19, SNP_20, and SNP_21 in both male (10 fish) and female (10 fish). Using the same RT-PCR and PCR products for sequencing, the amplified fragments of 551 bp of SNP_19 and SNP_20 were obtained. After T7EI digestion and analysis on a 2% agarose gel, male-specific SNP markers were clearly distinguished, as evidenced by the cleavage of amplicons into fragments of 175 bp and 374 bp (SNP_19, SNP_20) according to two-mismatch DNA base-pair cleavage (Figure 3.7). In contrast, female samples showed no digestion and retained intact products of 551 bp (Figure 3.7). In addition, when using both cDNA and gDNA templates, PCR amplification of SNP_21 yielded fragments of 520 bp (cDNA) and 518 bp (gDNA) in both sexes. Following T7EI digestion and electrophoresis on a 2% agarose gel, male-specific SNP markers were identified, as the amplicons were cleaved into fragments of 177 bp and 343 bp (cDNA) and 198 bp and 320 bp (gDNA) (Figure 3.7).

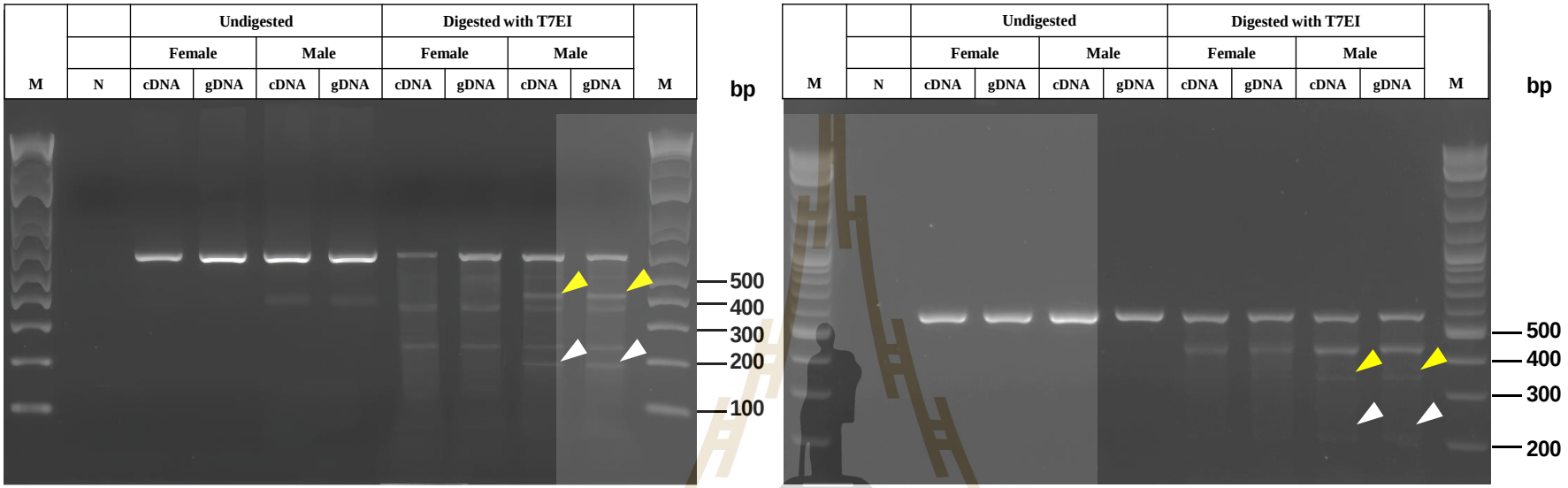

Figure 3.7 T7EI heteroduplex cleavage assay of three SNP-containing loci (SNP_19, SNP_20, and SNP_21) in snakeskin gourami. PCR products were amplified from purified cDNA and gDNA of female and male individuals and either left undigested or digested with T7EI. For SNP_19, 20 (left panel), both sexes exhibited a single intact band in the undigested samples, whereas specific cleavage fragments were detected only in male samples after T7EI digestion (indicated by yellow and white arrowheads), while female samples remained undigested. In contrast, the T7EI assay for SNP_21 (right panel) did not produce clear cleavage fragments (white arrowheads), indicating that the single-nucleotide mismatch at this locus could not be efficiently recognized by the enzyme.

Abbreviation: M = DNA marker; N = negative control.

3.5 Discussion

SNP-associated traits have been widely investigated for biological markers to identify variable traits displayed from genetic polymorphism, and transcriptome technology is a powerful tool for large-scale discovery of SNPs. Particularly in fish, when reference whole-genome sequencing data are limited, high-throughput RNA-seq analysis could be applied to survey a large number of SNPs at relatively low costs. For example, in yellow catfish (*Pelteobagrus fulvidraco*), RNA-seq analysis to identify differentially expressed genes (DEGs) between the ovary and testes also revealed 26,450 putative SNPs from approximately 1.2 million reads (Chen et al., 2015). In addition, in the green mud crab (*Scylla paramamosain*), transcriptome sequencing identified 13,271 SNPs from testis (171,962 reads) and ovary (193,154 reads) libraries (Gao et al., 2014). Likewise, in chum salmon (*Oncorhynchus keta*), 56,020 putative SNPs were obtained from 187,836 unigenes (Li et al., 2022). In this study, no reference genome is currently available for snakeskin gourami, whereas transcriptome data are available. Indeed, RNA-seq was employed to reveal sex-biased gene expression between testes and ovary in both juvenile and adult stages (Boonanuntanasarn et al., 2020; Jantawongsri et al., 2025). Using these databases, a total of 65,535 putative RNA SNPs associated with sex differences were discovered from juvenile (4 pools of 200 testes [JT1-4] vs 100 ovaries [JO1-4]) and adult (4 pools of 40 male [AT1-4] vs 40 female [AO1-4]), which generated 370,116,966 and 354,579,434 reads, respectively. According to a large number of reads and annotated unigenes (juvenile; 50,517 unigenes, adult; 55,883 unigenes), our transcriptome SNPs information was considered acceptable for further screening of sex-determination associated SNPs.

Practically, conventional Sanger sequencing has been implemented to validate and confirm SNP variants. For example, in the black-spotted frog (*Pelophylax nigromaculatus*), transcriptome analysis in testes and ovary revealed 35,406 RNA SNPs. Among these SNPs, 134 RNA SNPs which were in DEGs and consistent with male heterozygosity and female homozygosity were discovered. Subsequent DNA sequencing in gonadal tissues confirmed 83 sex-specific SNPs (Zhang et al., 2024). In addition, in the Chinese soft-shelled turtle (*Pelodiscus sinensis*), the gonadal RNA-seq revealed 35 novel polymorphic SNP markers from the comparative transcriptome of male and female (Li et al., 2022). Among these candidate SNPs, two were significantly

associated with sex phenotypes, with females being homozygous and males being heterozygous (Li et al., 2022). Furthermore, restriction-site associated DNA sequencing (RAD-seq) in mud crabs (*Scylla paramamosain*) identified 20 sex-specific SNPs (Shi et al., 2018). Subsequently, 10 SNPs were confirmed as heterozygous in females and homozygous in males by PCR with Sanger sequencing, demonstrating evidence for the ZW/ZZ sex determination system (Shi et al., 2018). In this study, filtering putative RNA SNPs using combination of databases from juvenile (JO vs JT SNPs) and adult (AO vs AT SNPs), 21 SNPs showed sex-specific RNA SNPs. Subsequently, RT-PCR with Sanger sequencing revealed 9 sex-specific RNA SNPs, with homozygous genotypes in females and heterozygous genotypes in males, providing a hypothesis for the XX/XY sex determination system in snakeskin gourami. Our findings were consistent with the previous findings, indicating an XX/XY mechanism in the snakeskin gourami (Panthum et al., 2021).

For large-scale SNP genotyping, PACE SNP assays provide a rapid, accurate, and high-throughput method for DNA variant genotyping assays, and thereby integrating into selective breeding programs (Torres et al., 2025). For example, in crop breeding, the utility of the PACE assay was demonstrated, as shown in the development of a robust SNP marker set for polyploid roses (Patzer et al., 2024). In Amazon Tambaqui (*Colossoma macropomum*), the practicality of PACE was applied to validate four potentially sex-specific SNPs from 14,805 SNPs in double digest restriction site-associated DNA (ddRAD) sequencing information, which showed 90.0 – 96.7 % association with the phenotypic sex (Varela et al., 2021). In line with these findings, the present study applied PACE genotyping assays to snakeskin gourami and revealed 9 sex-specific RNA SNPs, consistently showing homozygous and heterozygous genotypes across females and males, respectively. These results were in full concordance with Sanger sequencing, thereby confirming the reliability of PACE and highlighting its potential as a rapid and reliable approach for sex determination in fish populations.

Our *de novo* transcriptome assembly and annotation revealed that among nine RNA SNPs associated with sex determination, two SNPs are located in CDS (SNP_5 and SNP_21) of genes whose functions appeared to be crucial in sex differentiation. The nucleotide variant of SNP_5 is located in the CDS of *ezh2* and demonstrates a non-synonymous substitution with a change from lysine (K) to glutamic acid (E). This finding suggested that a change in an allele of AAG (K) to GAG (E) could be associated with sex

determination. In addition, *ezh2* showed significantly higher expression in adult females than in adult males, suggesting that its expression level might be involved in sex maturation. Consistent with this notion, studies in mammals have demonstrated that Ezh1/Ezh2-catalyzed H3K27-trimethylation regulates sex-biased gene expression in the liver, where loss of Ezh1/Ezh2 leads to the upregulation of female-biased genes in males and a concomitant disruption of sex-dependent transcriptional programs. Such differences may reflect lineage-specific adaptations of *ezh2*-mediated chromatin remodeling to reproductive development (Lau-Corona et al., 2020). These findings suggest a conserved role of *ezh2* in establishing sex differentiation across vertebrates, although the direction of regulation remains to be investigated in fish. The nucleotide variant of SNP_21 is found in the *kap3* CDS, although it demonstrates synonymous mutations. Indeed, a synonymous mutation was revealed to modulate translational efficiency and protein folding, thereby leading to affecting phenotype (Komar et al., 2024). In fact, *kap3* showed significant male-biased DEG in adult fish. A recent report has highlighted the critical role of kinesin motor proteins in spermatogenesis, particularly in the remodeling of Sertoli cell junctions, the restructuring of the blood–testis barrier, and the transport of spermatids and other organelles along the seminiferous epithelium in mammals (Yao et al., 2022). These processes are highly dependent on microtubule-based dynamics, for which kinesins act as key mediators and coordinators of germ cell movement and cell cycle progression. Therefore, our findings on sex-associated SNPs in *kap3l*, together with its male-biased expression, point to an important functional link between kinesin-associated proteins and sex differentiation in teleosts. Taken together, RNA variants in CDS together with differentially expressed levels in adults might be related to sex differentiation and sex maturation, respectively.

The 3′UTR of an mRNA molecule plays a crucial role in post-transcriptional gene regulation by controlling mRNA stability, translation efficiency, and subcellular localization (Hong and Jeong, 2023). Given these diverse regulatory roles, polymorphisms in 3′UTRs (SNP_2, SNP_3, SNP_19, and SNP_20) may drive sex-biased expression patterns and contribute to the molecular basis of sex differentiation. For example, SNP_2 is found at the 3’UTR of putative *ift172,* which produces a component of the intraflagellar transport complex. It was reported that *ift172* is essential for ciliary function, gamete motility, and sensory signaling in mice and humans (Zheng et al.,

2023). A variant of SNP_3 was found in the 3'UTR of *man2a1,* which encodes an α-mannosidase localized in the Golgi apparatus, which is critical for protein folding, stability, and function (Chu et al., 2012; Treccarichi et al., 2025). Its function was revealed to be important for germ cell survival, sperm maturation, and follicle development in oogenesis and spermatogenesis in mammals (Akintayo and Stanley, 2019). In addition, the nucleotide variants of SNP_19 and SNP_20 are found in the 3'UTR of *kap3,* which was revealed to play an important role in the transport of spermatids along the seminiferous epithelium in mammals (Yao et al., 2022). Note that nucleotide variants in *kap3* were observed in both the CDS and the 3'UTR. According to DEG analysis, *ift172, man2a1,* and *kap3* (adult only) exhibited a male-biased pattern. These findings could suggest that not only RNA variants, but also differential expression levels may contribute to male-biased reproductive processes.

Our results showed other RNA variants in SNP_4, SNP_12, and SNP_15, which were assembled and annotated as *crk*, *cs*, and *cast*, respectively. Among them, *crk* and *cs* (adult only) showed higher expression in the ovary than in the testes. The adaptor protein *crk* plays a conserved role in regulating cytoskeletal dynamics and cell fate (Shi et al., 2023), although its function related to sex differentiation remains to be elucidated. Citrate synthase (CS), a core enzyme of the tricarboxylic acid (TCA) cycle, has distinct roles in reproduction beyond its mitochondrial function. In females, *cs* is important in the *de novo* synthesis of fatty acids and membrane lipids (Verschueren et al., 2019). Differential expression of *cs* may act as a sex-biased metabolic regulator, potentially supporting the high energy demands of oogenesis. In males, the extra-mitochondrial isoform (*eCS*), abundantly localized in the sperm head, is crucial for initiating the first spike of Ca^{2+} oscillation in the initial calcium spike for activating the egg for proper fertilization (Kang et al., 2020). Although the *cast* did not show differential expression between males and females, its function was crucial in both male and female reproductive systems, particularly in sperm and oocytes. In the calpain-calpastatin system, calpastatin contributes to the calcium-dependent acrosome reaction, which is a critical step for sperm to penetrate the oocyte in mammals (Yudin et al., 2000). In oocytes, the calpain-calpastatin system plays a role in calcium-mediated processes during oocyte activation in humans (Ben-Aharon et al., 2005). In livestock, SNPs in the *cast* were linked to several reproductive traits (Gábor et

al., 2012). Taken together, nucleotide variants in the 3'UTR is several genes that are crucial in the reproductive system could have an important role in sex determination.

In addition to RNA-level variation, three of the nine sex-specific SNPs (SNP_19, SNP_20, and SNP_21) were consistently validated at the gDNA level, including PCR followed by Sanger sequencing, PACE genotyping assays, and the T7EI mismatch cleavage assay. The fish population of SUT1, SUT2, SUT3, and SUT4 was originally collected from commercial farms and natural water sources in the Central and Northeast parts of Thailand. The presence of sex-related SNP_19, SNP_20, and SNP_21 in multiple fish populations suggests that these markers are not confined to sex identification in the experimental population alone. Nevertheless, additional population-level validations are necessary to confirm their utility as sex-specific markers. Note that these 3 SNPs are located in *kap3l*, which was revealed to play a critical role in spermatogenesis and the transport of spermatids in mammals (Yao et al., 2022). The Sanger sequencing demonstrated complete concordance and exhibited a clear genotypic dimorphism: homozygous in females and heterozygous in males in the snakeskin gourami. Their presence in both transcriptomic and genomic datasets confirms their concordance with sex-specific SNP markers. In addition, PACE SNP assays exhibited male heterozygous and female homozygous genotypes, providing high-throughput, accuracy, reliability, and versatility for further marker-assisted sex identification applications in fish populations. T7EI is a mismatch-sensitive nuclease that cleaves heteroduplex DNA at sites of base-pair mismatches or small insertions/deletions, making it a cost-effective and widely useful method for SNP and mutation validation (Vouillot et al., 2015). This study employed the T7EI assay to detect DNA heteroduplex according to male heterozygous genotypes of SNP_19 with SNP_20 and SNP_21 for both cDNA and gDNA, confirming the reliability of the T7EI assay in discriminating between genotypic sexes in snakeskin gourami. However, the T7EI assay for the single-nucleotide mismatch of SNP_21 could not give clear digested bands. It was revealed that the T7EI assay is capable of identifying indels larger than 2 bp, while it has limited sensitivity to single-nucleotide mismatches (Bhattacharya and Van Meir, 2019). Collectively, the consistent outcomes across three independent methodologies and various fish populations strongly support that *kap3l*-linked SNPs represent gDNA-based SNPs as reliable sex-linked markers, demonstrating their utility for genetic sex identification in snakeskin gourami.

Our results showed that, using both the Sanger sequencing and PACE analysis of DNA variants in gDNA, six SNPs (SNP_2, SNP_3, SNP_4, SNP_5, SNP_12, and SNP_15) did not exhibit a heterozygous genotype in males. These findings suggested that there might be post-transcriptional processes during sex differentiation in male fish. Although Sanger sequencing of cDNA revealed mixed peaks (e.g., A/G) at SNPs_5, SNPs_12, and SNPs_15, the subsequent PACE genotyping on genomic DNA did not consistently yield discrete male/female cluster separation. This discrepancy can be attributed to the nature of A-to-I RNA editing, which commonly occurs at partial editing frequencies rather than complete conversion. For instance, several human and mammalian studies report editing efficiencies of < 20% at the majority of sites (Chalk et al. 2019) and only ~10–50% even at relatively efficient loci (Daniel et al. 2017). In addition, the editing index across transcriptomes often reveals substantial numbers of partially edited transcripts (< 1% in some contexts; Bazak et al. 2014). Because the PACE assay is optimized to distinguish discrete allele-specific genotypes (i.e., homozygous vs heterozygous) rather than finely quantify editing ratios, samples in which only ~10–60 % of transcripts carry the edited form will produce intermediate fluorescence signals and result in cluster overlap or unclear segregation. These overlapping clusters thus reflect incomplete editing rather than assay failure. In the context of our male-biased editing loci, the higher ADAR activity in males may increase but not guarantee 100 % editing, resulting in a spectrum of edited transcripts that complicates allele discrimination by PACE. Ultimately, these findings underscore that PACE genotyping of cDNA/edited transcripts should be interpreted with caution when editing rates are moderate or variable, and that Sanger or other quantitative assays like deep sequencing may better capture editing proportions.

To further interpret this observation at the molecular level, one plausible mechanism involves adenosine-to-inosine (A-to-I) RNA editing, a well-documented post-transcriptional modification catalyzed by adenosine deaminases acting on RNA (ADARs), which convert adenosine residues into inosine that is interpreted as guanosine during translation (Nishikura, 2016; Snyder et al., 2017). Our results showed that *adar* exhibited upregulation in the testes compared with that in the ovary in both juveniles and adults. The male-biased *adar* expression would elevate RNA editing activity in males, which may contribute to male-specific RNA variants, even in the absence of corresponding genomic polymorphisms. Previous studies in the olive flounder (*Paralichthys olivaceus*) revealed

extensive A-to-I RNA editing, predominantly occurring in the 3′UTRs of transcripts, with higher editing frequencies in testes than ovaries, consistent with elevated *adar1*, *adar2*, and *adar3* expression in the testis (Wang et al., 2020). Moreover, A-to-I editing is known to be highly active during early embryogenesis, when development depends on maternal mRNAs and proteins, as demonstrated in zebrafish (Buchumenski et al., 2021). Together, these findings suggest that RNA editing represents a key regulatory mechanism underlying sexual differentiation, particularly in heterogametic males, and may also explain the discrepancies observed between RNA and DNA variants.

3.6 Conclusion

In summary, transcriptome-based SNP discovery in juvenile and adult snakeskin gourami identified nine RNA SNPs located in *ift172, man2a1, crk, ezh2, cs, cast,* and *kap3l*, with differential expression between testes and ovaries, suggesting that both nucleotide variants and expression levels are associated with sex differentiation. These RNA SNPs exhibited heterozygosity in males and homozygosity in females, supporting an XY sex-determining system in *T. pectoralis*. Among the nine RNA SNPs, three exhibited concordant nucleotide variants at the genomic level, demonstrating their potential as reliable markers for genotypic sex identification. The absence of genomic variants in the remaining six RNA SNPs, together with male-biased expression of *adar,* indicates that RNA editing may play a regulatory role in the process of sex differentiation. Furthermore, a mismatch-sensitive T7EI assay confirmed the sex-associated nucleotide variants at SNP_19 and SNP_20. Collectively, these findings provide novel molecular evidence for the genetic and post-transcriptional regulation of sex differentiation and offer promising markers for sex identification and selective breeding in the snakeskin gourami.

3.7 Acknowledgements

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3.8 References

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CHAPTER IV

Validation of XX/XY Sex Determination and All-Female Production Via 17α-Methyltestosterone–Induced Neomales in Snakeskin Gourami (*Trichopodus Pectoralis*)

4.1 Abstract

The snakeskin gourami (*Trichopodus pectoralis*) exhibits pronounced female-biased sexual size dimorphism, making all-female production highly desirable for aquaculture. The use of neomale broodstock represents an indirect strategy for achieving monosex populations while avoiding hormone application during grow-out. This study aimed to evaluate the effectiveness of 17α-methyltestosterone (MT)-induced sex reversal for neomale production, genetic sex identification, and reproductive performance in snakeskin gourami. Sex reversal was induced by feeding fry with an MT-supplemented diet (MT-100) from 7 to 97 days post-hatching (dph), while control fish received a basal diet. Sex-specific SNP markers were applied to distinguish homozygous females (XX), heterozygous males (XY), MT-treated males (XY), and MT-derived neomales (XX). Gonadal development and reproductive performance were assessed using squash methods, histological analysis, progeny testing, and controlled breeding experiments. Although MT-treated male broodstock exhibited reduced reproductive performance compared with control males, they retained the ability to produce functional sperm and achieve successful fertilization. Crosses between control males or MT-treated males and wild-type females produced progeny with an approximately 1:1 sex ratio. In contrast, crosses between MT-derived neomales (XX) and females (XX) consistently produced 100% female offspring. Genotypic analysis of progeny using PACE SNP assays confirmed that neomales transmitted the XX genotype. Collectively, these findings demonstrate that MT-induced neomales function as phenotypic males and provide strong evidence that snakeskin gourami follows an XX/XY sex determination system. This study establishes an integrated framework combining hormonal sex reversal, molecular marker-assisted genotyping, and neomale-

based breeding, providing a practical and sustainable strategy for hormone-free all-female production in female-biased aquaculture species.

Keywords: snakeskin gourami, 17α-methyltestosterone, sex reversal, neomales, all-female production.

4.2 Introduction

Among vertebrates, sexual dimorphism is widely found in teleosts, which occasionally influences traits of commercial significance, such as growth rate, metabolic and physiological parameters, and external morphology (Devlin and Nagahama, 2002; Liu et al., 2024). For example, growth-related sexual dimorphism is observed in Nile tilapia (*Oreochromis niloticus*), zig-zag eel (*Mastacembelus armatus*), and red Tilapia (*Oreochromis spp.*), in which males grow faster than females (Asad et al., 2024; Lu et al., 2024; Yostawonkul et al., 2023). In contrast, mature females are larger than males in species such as the large yellow croaker (*Larimichthys crocea*), rainbow trout (*Oncorhynchus mykiss*), and snakeskin gourami (*Trichopodus pectoralis*) (Kabpha et al., 2023; Mu et al., 2024; Van Der Honing, 2001). In addition, plasma metabolites, hematology, and immune parameters revealed clear sex-specific differences in Nile tilapia, snakeskin gourami, yellowtail kingfish (*Seriola lalandi*), Brown trout (*Salmo trutta fario*), and giant kokopu (*Galaxias argenteus*), which showed distinct physiological profiles between males and females (Phumyu et al., 2012; Kabpha et al., 2023; Lulijwa et al., 2021; Marijani et al., 2017; Sheikh et al., 2022; Valero et al., 2024). Indeed, it was demonstrated that sex-related phenotypic differences in fish are obvious during adulthood, particularly in the maturation stage, suggesting that sex hormones would be mainly involved in sexual dimorphism (Kawajiri et al., 2015). Furthermore, sexually dimorphic gene expression in the gonads has been demonstrated in both juvenile and adult stages demonstrating that several genes are involved in sex determination and differentiation snakeskin gourami, Tongue Sole (*Cynoglossus semilaevis*), loach, half-smooth tongue sole, yellow catfish, and Nile tilapia (Boonanuntanasarn et al., 2020; Jantawongsri et al., 2025; Luo et al., 2022; Wang et al., 2016). Therefore, sexual dimorphic traits and their regulation in fish have been

intensively studied not only for improving aquaculture-related traits but also for advancing fundamental studies on genetic sex determination and physiological mechanisms of sex differentiation (Baroiller and D'Cotta, 2001; Piferrer et al., 1994).

Sex differentiation in fish is highly plastic, being influenced by genetic factors and environmental cues such as exogenous hormones and population density (Guiguen et al., 2010; Heule et al., 2014; Okutsu et al., 2015). Indeed, several oviparous fish offer sex reversal manipulation during the larval stage (at the labile period of gonad development). Particularly, exogenous sex hormone treatment influencing sex reversal can be manipulated during early gonadal development through the administration of exogenous androgens, and estrogen, such as 17α-methyltestosterone (MT), and estrogen, such as 17β-estradiol (E2), respectively, in fish (El-Greisy and El-Gamal, 2012; Liu et al., 2021, 2024; Mu et al., 2024; Teal et al., 2024; Universidade Federal de Santa Catarina et al., 2018). In addition, sex reversed fish exhibited physiological appearances similar to their naturally sexual traits. For instance, in Nile Tilapia, dietary MT treatment can produce a high proportion (~95%) of male populations, and the resulting sex-reversed males exhibit growth performance and physiological characteristics equivalent to those of naturally males (Asad et al., 2024). Similarly, E2 feminization in green sunfish (*Lepomis cyanellus*) and red shiner (*Cyprinella lutrensis*) produced fully functional phenotypic females, which showed similar survival rate, growth, and ovary development to those of control females (Teal et al., 2024, 2023). Moreover, the cryopreserved semen of MT sex-reversed males showed fertilization rates comparable to those of normal males, demonstrating that sex-reversed males in rainbow trout possess equivalent reproductive performance to genetic males (Ninhaus-Silveira et al., 2006). Therefore, sex reversal using exogenous sex hormone treatment has been applied in fish to gain sexual benefits for a monosex population in aquaculture-related fish.

The snakeskin gourami is a commercially important freshwater species native to South Asia. FAO statistics indicate that global production reached approximately 30,500 tonnes in 2021, with Thailand being a leading contributor (Sutthakiet et al., 2024). From an economic perspective, the female snakeskin gourami grows approximately 1.2–1.3 times faster than males under intensive culture (Kabpha et al., 2023; Panthum et al., 2021). This sexual dimorphism in growth highlights the advantage of producing all-female stocks for aquaculture productivity. It was demonstrated that dietary

supplementation of E2 feeding to snakeskin gourami fry could produce an all-female population, which proved highly responsive to hormonal manipulation, and the resulting sex-reversed females exhibit fast growth and physiological characteristics resembling those of control females. (Kabpha et al., 2023). A previous study in our laboratory aimed to evaluate the effects of MT-induced sex reversal on sex ratio, growth performance, several physiological parameters, including blood metabolites, hematological indices, and immune response. We demonstrated that MT-100 was optimum for the production of sex reversed male. Also, MT-induced males and control males exhibited similar gene expression profiles in the testes, whereas both groups showed differentially expressed genes compared with the female ovary. An indirect strategy for producing all-female populations in fish with an XX/XY sex determination system involves the use of neomale broodstock crossed with normal females (Figure 4.1A). This approach has attracted increasing interest as a potential alternative to direct hormonal treatments during grow-out stages. However, the practical application of this strategy requires reliable tools for the identification of genetic sex and the validation of sex-reversed individuals. In our previous study, sex-specific DNA markers were developed, which were able to identify the genotype of homozygous females and heterozygous males, and proposed its XX/XY sex determination system (Chapter III). Therefore, this study aimed to use sex-specific DNA markers for genetic sex identification in snakeskin gourami and to assess the potential of neomale broodstock in monosex breeding programs. By integrating molecular sex identification with controlled breeding strategies, this work seeks to provide a practical and sustainable approach for hormone-free all-female production in fish species with an XX/XY sex determination system.

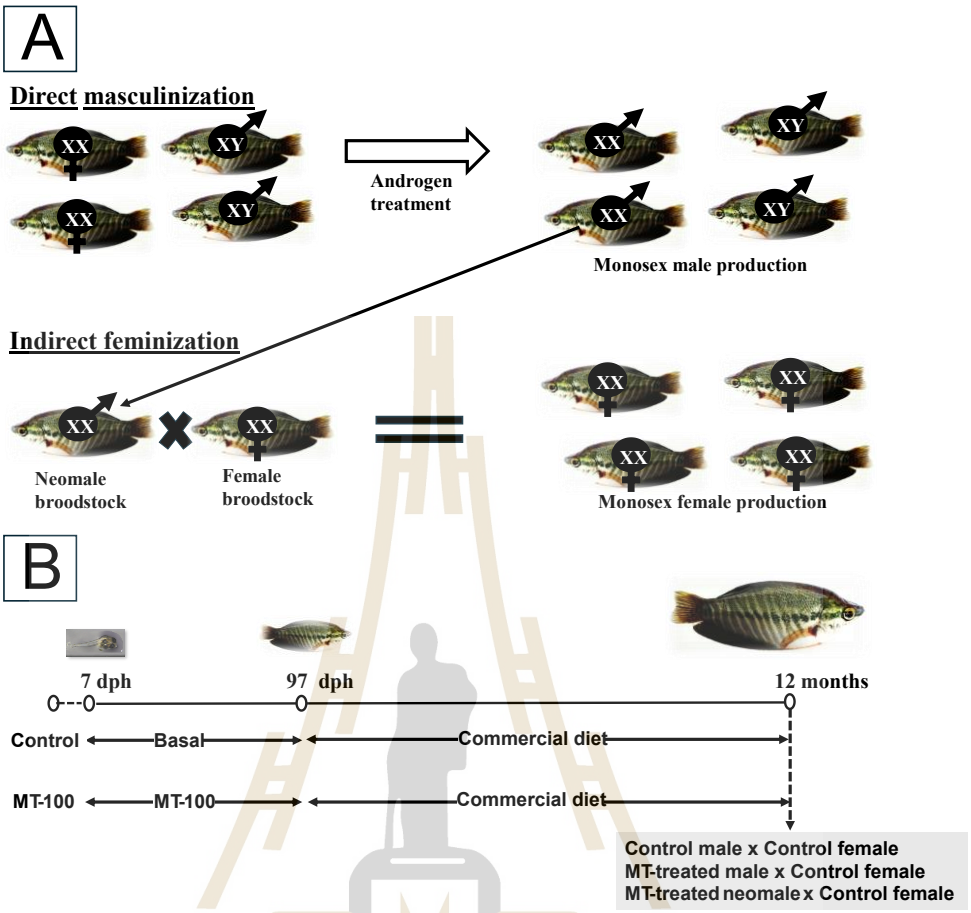

Figure 4.1 Experimental plan for MT-induced neomale production in snakeskin gourami. 7-dph fry were fed a basal diet (control) or MT-100 for 90 days. Subsequently, the fingerlings were cultured through mature stage to become bloodstock. Control male, MT-treated male and MT-treated neomale were mated with control female, and the progeny were cultured for 3 months for sex determination. Using SNP_19, SNP_20 and SNP_21, genotypes of control male, MT-treated male and MT-treated neomale were identified using Sanger sequencing and PACE SNP. Sex determination in progeny was performed using squash method and histological analysis. PACE SNP analysis of SNP_19, SNP_20 and SNP_21 was conducted to identify genotypes of progenies.

4.3 Materials and methods

4.3.1 Ethics statement

All protocols related to the experimental fish in this study were approved by the Ethics Committee of the Suranaree University of Technology Animal Care and Use Committee (approval no. 1731039).

4.3.2 Fish preparation

The snakeskin gourami was obtained from commercial farms in Samut Sakhon and subsequently transported to and maintained at the Suranaree University of Technology (SUT) farm in Nakhon Ratchasima, Thailand. Fish were acclimated before experimentation and reared in a 0.04-hectare earthen pond under ambient photoperiod conditions in freshwater. During the culture period, fish were fed to apparent satiation twice daily (10:00 and 16:00) with a commercial formulated diet (Aquafeed, Thai Union Feedmill, Samut Sakhon, Thailand) containing 40% crude protein and 6% lipid.

4.3.3 Neomale broodstock production

The experimental plan for neomale production in this study is shown in figure 4.1B. Fish breeding (1 male: 1 female) was conducted in an aerated concrete pond (2 m × 2 m × 1 m) containing floating aquatic vegetation to support bubble nest formation. Spawning was induced by hormonal injection. Female broodstock received two intramuscular injections: an initial priming dose of 10 μg kg^{-1} body weight of luteinizing hormone-releasing hormone analog (LHRHa; Suprefact, Hoechst, Germany) combined with 5 mg kg^{-1} domperidone (Motilium-M, OLIC, Bangkok, Thailand), followed 12 h later by a resolving dose of 10 μg kg^{-1} LHRHa and 5 mg kg^{-1} domperidone. Male broodstock received a single intramuscular injection of 10 μg kg^{-1} LHRHa combined with 5 mg kg^{-1} domperidone. Fertilized eggs were observed after 14 hours post-spawning, and the broodstock were subsequently removed. Larvae were reared in the same tanks until 6 dph. The offspring were divided into two ponds for control and MT-reversed fish. The control fry was fed with a free-MT diet while the MT-reversed fish were fed with the MT-100 diet until three months. Subsequently, 200 fish were transferred into hapas (2 × 2 × 2 m), which were set up in an earthen pond and fed with the commercial diet (32% CP and 4 % fat) through the mature stage (12 months).

4.3.4 Progeny testing

Progeny testing was conducted using three mating groups: (i) control males crossed with wild-type (WT) females, (ii) MT-treated males paired with WT females, and (iii) MT-derived neomales crossed with WT females. For the control group, a total of 36 male broodstock were selected for breeding with WT females, hormonal induction using LHRHa and domperidone, as previously described. Among these, 27 males successfully produced offspring. Fin clips were collected from six control males and six control female broodstock for genotyping, and their progeny were reared for subsequent sex determination.

In the MT-100 treatment group, 40 MT-treated males were selected for breeding with WT females using the same hormonal induction protocol. Of these, 13 males successfully produced offspring, and fin samples were collected from all reproductively active individuals for genotyping. A total of seven progeny populations were obtained from MT-treated males and reared for sex determination at three months post-hatching. In addition, six MT-treated males were identified as neomales, and their progeny were also cultured for sex determination.

Fish breeding was conducted in mating tanks (2 m × 2 m × 1 m) with gentle aeration. Fourteen hours after spawning, the broodstock were removed from the tanks to prevent egg and larval predation. Larvae were initially reared in the spawning tanks. Subsequently, larvae (7 dph) were fed a commercial diet containing 40% protein and 8% fat until 30 dph. Thereafter, fingerlings from all experimental groups were transferred to 2 × 2 × 2 m^3 hapas installed within a 0.08-ha earthen pond. Fish were hand-fed a commercial diet containing 35% protein and 4% lipid for a further three months. At 97 dph, individuals were randomly sampled for fin tissue collection for genotyping, and gonads were collected for sex determination using the squash method (Menu et al., 2005) and histological examination. Throughout the rearing period, ambient air temperatures ranged from 22.0–35.0°C, while water temperatures were maintained between 28.5–30.0°C.

4.3.5 Histological examination of the gonads

Sex determination was performed through histological examination of gonadal tissue. Samples were fixed in Bouin's solution at 4°C for 16 hours. Sex ratio was determined in fish at 3 months of age (n = 60 fish per replicate; six replications). Followed by dehydration through a graded ethanol series (70% to 100%). The tissues were then embedded in paraffin, sectioned at 5 µm, and mounted on glass slides. Sections were subsequently dewaxed, rehydrated through xylene and ethanol series, and stained with hematoxylin and eosin (H&E). Gonadal morphology was examined using a digital upright microscope (Olympus BX53, Tokyo, Japan).

4.3.6 Genotypic analysis using sex-specific SNP

Based on a previous study, sex-specific SNP markers (SNP_19, SNP_20, and SNP_21) were developed to distinguish heterozygous males and homozygous females in snakeskin gourami (Chapter III). In the present study, Sanger sequencing and PACE SNP assays were employed to determine the genotypic sex of control males, MT-treated males, MT-derived neomale broodstock, and their offspring. Genomic DNA (gDNA) was extracted from fin tissue (approximately 100 mg) using the standard phenol-chloroform method. Briefly, fin samples were minced and incubated in lysis buffer containing proteinase K at 55°C until complete tissue digestion was achieved. Following lysis, an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1, v/v/v) was added, and the mixture was gently vortexed and centrifuged to separate the aqueous and organic phases. The upper aqueous phase containing DNA was carefully transferred to a new tube and further extracted with chloroform to remove residual phenol. gDNA was subsequently precipitated by the addition of cold absolute ethanol in the presence of sodium acetate, followed by centrifugation. The resulting DNA pellet was washed with 70% ethanol, air-dried, and resuspended in TE buffer. DNA concentration and purity were assessed using a NanoDrop™ ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) before downstream applications. Primer sequences, annealing temperatures, and expected amplicon sizes are presented in (Table 4.1). PCR amplification was carried out using TaKaRa Ex Taq polymerase (TaKaRa Bio, Shiga, Japan) in a total reaction volume of 25 µL, consisting 2.5 µL 10 × Ex Taq Buffer, 2.0 µL dNTP Mixture (2.5 mM each), 0.125 µL Ex Taq polymerase (5 U μL^{-1}), 2.5 µL each of forward and reverse primers (2 µM), 2.5 µL gDNA template (50ng μL^{-1}), and 12.875 µL nuclease-

free water. PCR was performed under the following cycling conditions: initial denaturation at 95°C for 3 min; 35 cycles of 95°C for 30 s, annealing at the primer-specific optimal temperature (55–65°C) for 30 s, and extension at 72°C for 25 s; followed by a final extension at 72°C for 10 min. The target PCR products were excised from agarose gels and purified using the QIAEX® II Gel Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Purified products were subjected to Sanger sequencing to identify nucleotide variation at the target SNP locus.

PACE SNP assays (PCR Allele Competitive Extension) were employed as a high-throughput genotyping approach, in which allele-specific fluorescent probes competitively extend during PCR, enabling rapid and accurate genotype discrimination based on fluorescence signal patterns. SNP genotyping was performed using the PACE® 2.0 Genotyping Master Mix (3CR Bioscience, Harlow, UK) according to the manufacturer's instructions. Each reaction in a total volume of 5 µL, containing 2.5 µL of 2× PACE 2.0 Genotyping Master Mix, 0.1 µL of custom-designed Assay Mix (comprising two allele-specific forward primers at 12 µM each and one common reverse primer at 30 µM), and 20 ng of gDNA template. PCR amplification was performed under the following cycling conditions: initial enzyme activation at 94°C for 15 min; followed by 10 cycles of 94°C for 20 s and 65–57°C for 60 s with a touchdown of 0.8°C per cycle; followed by 30 cycles of 94°C for 20s and 57°C for 60s. Following amplification, endpoint fluorescence signals of FAM and HEX were measured at below 40°C using a LightCycler® 480 real-time PCR platform (Roche, Germany). Genotypes were assigned based on relative FAM and HEX fluorescence intensities, and allelic discrimination cluster plots were generated for genotype calling. DNA-free reactions were included as negative controls, which exhibited no detectable fluorescence, confirming the absence of cross-contamination.

4.3.7 Data analysis

All statistical analyses were performed using SPSS version 22.0 (SPSS Inc., Chicago, IL, USA). Chi-square (χ^2) tests were used to assess deviations from the expected 1:1 sex ratio in MT-treated and control groups and to compare sex ratios among progeny derived from control males, MT-treated males, and MT-treated neomales. A P-value < 0.05 was considered statistically significant.

Table 4.1 Sequences of primers used for Sanger sequencing and PACE genotyping.

Name	Primers (5′-3′)	Tm (°C)	Products (bp)	Application
SNP_19, 20	F: AATACGACTCACTATAGGGCCAGGGCTGTAACTCTGGACT R: GCACCTTGATCAAAGCACTCTCCA	63	551	Sanger sequencing
SNP_21	F: GAGACAGCTCTAGGGGCTCT R: TGCTCCTGTTTGGCCAGAAG	60	518	Sanger sequencing
SNP_19, 20 (PACE)	CRP:CTACTAATGCATTAAATGTCCTAAGCTCCC ASP-GC:GAAGGTGACCAAGTTCATGCTAATATTGAGCATTTACAATACTGGACATGC ASP-TT:GAAGGTCGGAGTCAACGGATTAATATTGAGCATTTACAATACTGGACATTT	60	100	PACE genotyping
SNP_21 (PACE)	CRP:ATAGTGTGGAGCTGGCCACCATTAT ASP-G:GAAGGTGACCAAGTTCATGCTTATAATGACTCACCACTCCATGAAAC ASP-A:GAAGGTCGGAGTCAACGGATTTTATAATGACTCACCACTCCATGAAAT	60	195 (ASP-G) 196 (ASP-A)	PACE genotyping

Abbreviation: Tm (°C) =Annealing temperature (°C); bp= base pair; ASP=Allele-specific forward primers; CRP=Common reverse primer; PACE=PCR Allele Competitive Extension.

Note: The underlined sequence AATACGACTCACTATAGGG represents the T7 universal sequencing tag added to the 5′end of primers. The universal FAM (green) and HEX (orange) fluorescent tails were appended to the 5′ends of allele-specific primers for PACE genotyping.

4.4 Results

4.4.1 Genotypic identification of control males, MT-treated males, MT-derived neomales, and females broodstock by Sanger sequencing

Table 4.2 shows that among 36 selected mature control males, 27 individuals successfully produced offspring, corresponding to a mating success rate of 75.0%. In contrast, only 13 of 40 MT-treated males (32.5%) were able to produce offspring. For progeny testing, six offspring from each mating group, including control males, MT-treated males, and MT-treated neomales, were selected and reared for 90 days for subsequent sex ratio determination. The genotypes of all broodstock, including control males, MT-treated males, and MT-derived neomales and females, were determined using PCR amplification followed by Sanger sequencing. These results were consistent with previous findings indicating that snakeskin gourami possesses an XX/XY sex determination system (Chapter III). Genotyping results showed that control males and MT-treated males were heterozygous at the SNP_19 (G/T), SNP_20 (C/T), and SNP_21 (G/A; complementary T/C detected in antisense sequencing), whereas MT-derived neomales and females were homozygous at the SNP_19 (G/G), SNP_20 (C/C), and SNP_21 (G/G) loci (Figure 4.2, 4.3, and 4.4).

Table 4.2 Number of control male and MT-treated male broodstock that were used for progeny testing.

Parameters	Control group		MT-100 group	
	Males	females	males	Control females
No. of broodstock	36	36	40	40
No. of pairs with hatching	27	27	13	13
No. of pairs with no hatching	9	9	27	27
Mating success (%)	75.0	75.0	32.5	32.5

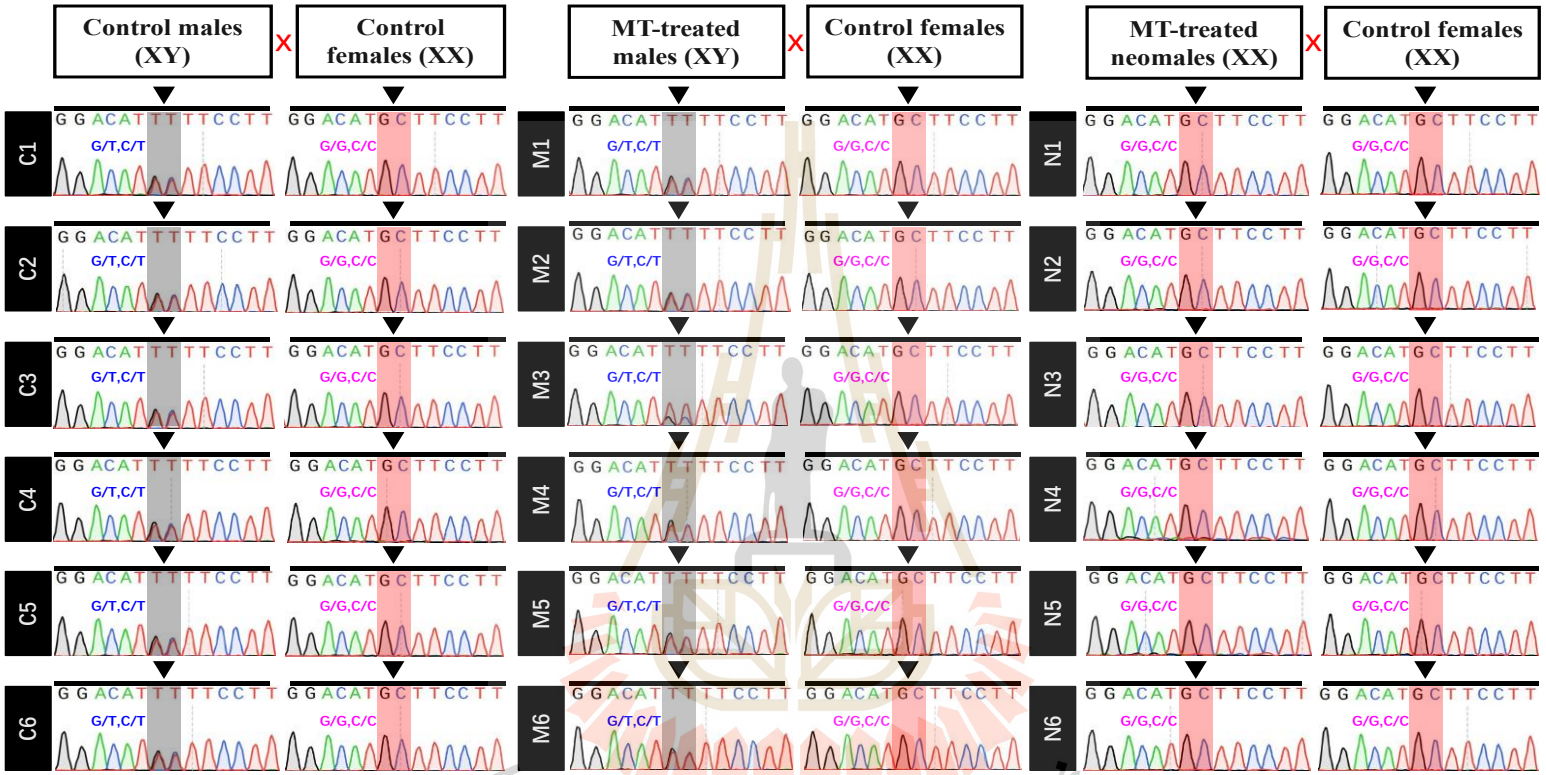

Figure 4.2 Identification of sexual genotypes at SNP_19 and SNP_20 in broodstock using Sanger sequencing. Representative chromatograms from control males × control females (C1–C6), MT-treated males × control females (M1–M6), and MT-treated neomales × control females (N1–N6) showed that control males and MT-treated males were consistently heterozygous at SNP_19 (G/T) and SNP_20 (C/T), whereas control females and MT-treated neomales were homozygous at both loci (G/G and C/C). Abbreviations: C = control group; M = MT-treated male; N = MT-treated neomale.

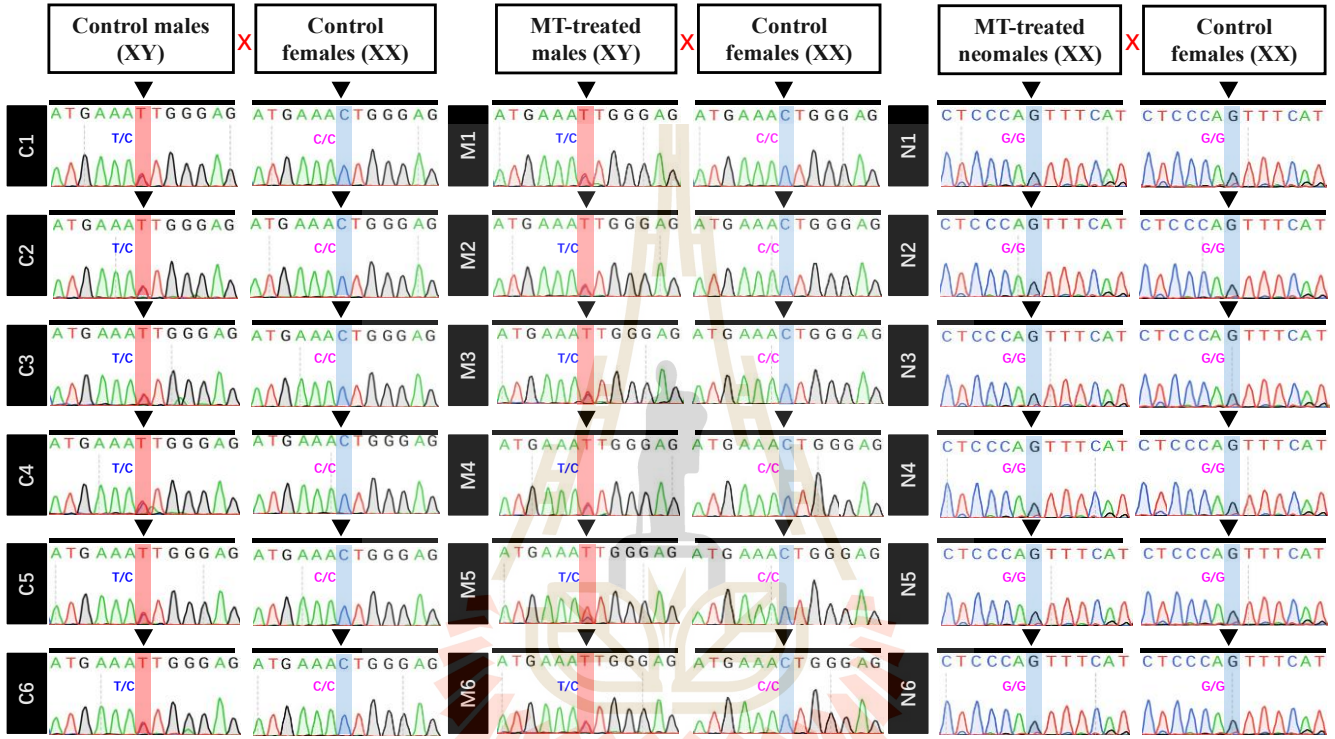

Figure 4.3 Identification of sexual genotypes at SNP_21 in broodstock using Sanger sequencing. Representative chromatograms from control males × control females (C1–C6), MT-treated males × control females (M1–M6), and MT-treated neomales × control females (N1–N6) showed that males were consistently heterozygous at SNP_21 (T/C, complementary to G/A), whereas females and neomales were homozygous (C/C, complementary to G/G). Abbreviations: C = control group; M = MT-treated male; N = MT-treated neomale.

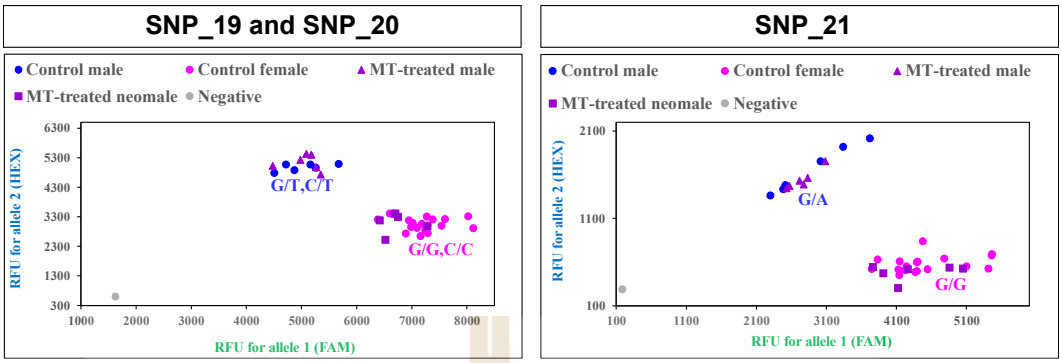

Figure 4.4 Genotypic validation of sex-specific SNPs (SNP_19, SNP_20, and SNP_21) in broodstock using PACE assays. Scatter plots illustrate relative fluorescence unit (RFU) signal intensities for allele 1 (FAM, x-axis) and allele 2 (HEX, y-axis). Each point represents an individual fish (pink = female; blue = male; purple = MT-treated male and MT-treated neomale; gray = negative control). SNP_19, SNP_20, and SNP_21 showed homozygous females and heterozygous males.

4.4.2 Genotypic identification of control males, MT-treated males, MT-treated neomales, and females using PACE genotyping

Consistent with the genotyping results obtained by Sanger sequencing, broodstock genotypes (control males, MT-males, MT-neomales, and females) were further validated using PACE SNP assays. The PACE genotyping results clearly discriminated genotypic classes among broodstock groups. Specifically, control males and MT-treated males consistently exhibited heterozygous genotypes. In contrast, MT-treated neomales and females displayed homozygous genotypes at the SNP_19 (G/G), SNP_20 (C/C), and SNP_21 (G/G) loci (Figure 4.4). No discordance was observed between genotypes determined by PACE SNP assays and those obtained by Sanger sequencing, confirming the robustness and reliability of the PACE-based genotyping approach for genetic sex identification in snakeskin gourami.

4.4.3 Progeny testing of control males, MT-treated males, and MT-derived neomales using the squash method and histological analysis

Using the squash method, the gonadal sex of progeny was preliminarily assessed based on cellular morphology. As shown in (Figure 4.5A and B), ovarian and

testicular tissues were clearly identified in female and male progeny, respectively. To validate the accuracy of the squash method, histological analysis was subsequently performed on corresponding samples, confirming ovarian structures in female progeny and testicular structures in male progeny. In female progeny, squash preparations revealed numerous rounded oocytes with distinct cellular boundaries (Figure 4.5A), which closely corresponded to the ovarian morphology observed in histological sections. Histological examination showed ovaries containing oogonia (OG) and previtellogenic oocytes (PVO), indicative of immature ovarian development (Figure 4.5 D, F, G, and H). In male progeny, the squash method displayed dense aggregates of spermatogenic cells, predominantly spermatocytes (Figure 4.5B). Consistently, histological sections revealed well-organized lobular testes containing spermatogonia (SG), primary spermatocytes (PSC), secondary spermatocytes (SSC), and a small proportion of spermatids (ST) distributed throughout the testicular tissue (Figure 4.5C and E). Similar gonadal characteristics were observed in progeny derived from control males, MT-treated males, and MT-derived neomales, all of which exhibited representative features of immature ovaries or testes at the juvenile stage (Figure 4.5C–H). The strong concordance between squash preparations and histological observations demonstrates that, by 3 months post-hatching, gonadal sex in snakeskin gourami progeny can be reliably distinguished based on distinct gonadal morphology. These results confirm the accuracy and practicality of the squash method as a rapid and efficient tool for early sex determination and large-scale progeny screening.

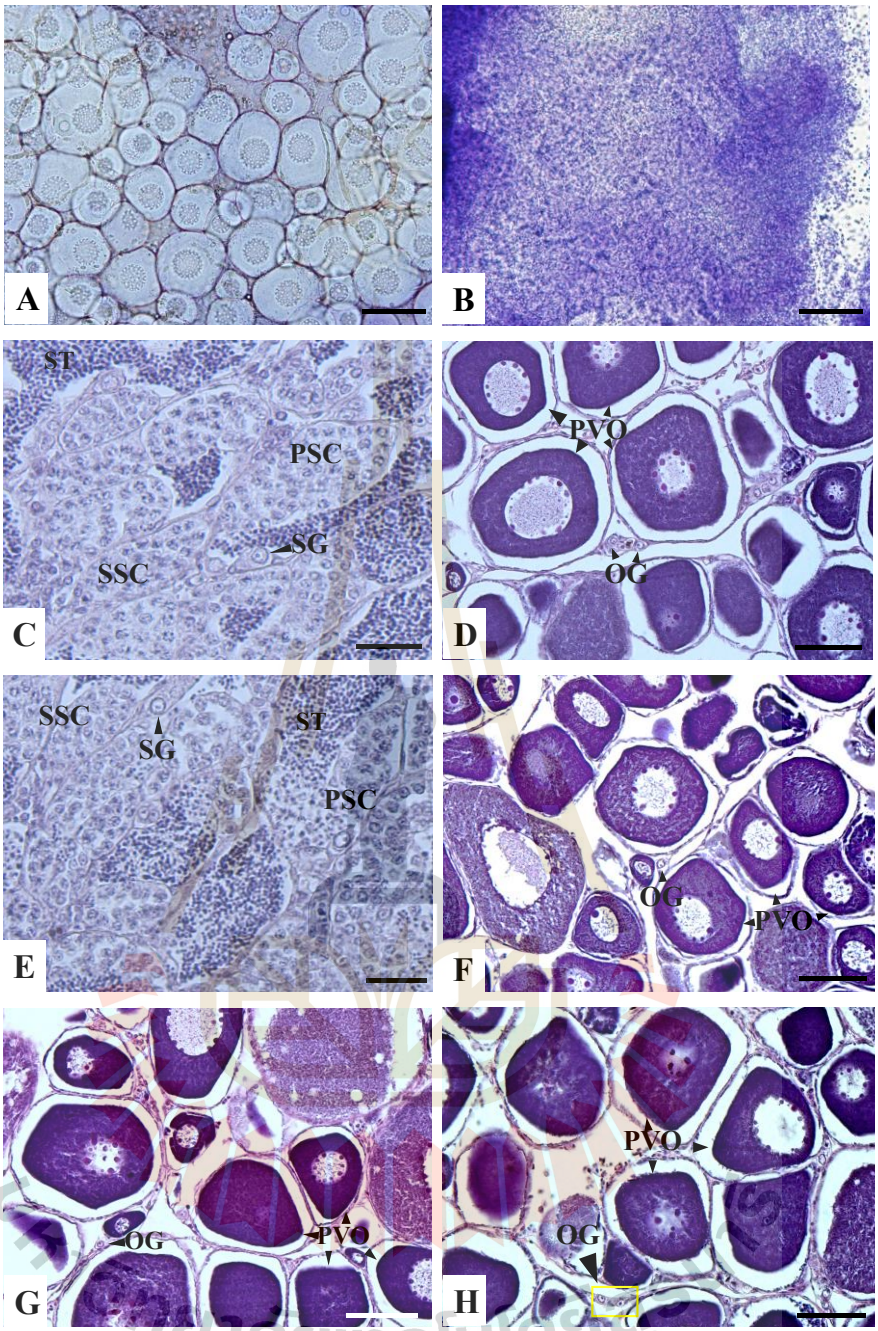

Figure 4.5 Histological and squash-based characterization of gonadal development in progeny derived from neomale experiments in snakeskin gourami. (A) ovary of fingerlings at three months determination using squash method. (B) testis of fingerlings at three months of determination using the squash method. (C) testis of fingerlings at three months of determination using histological analysis in the control group offspring. (D) ovary of fingerlings at three months using histological analysis in the control group offspring. (E) testis

of fingerlings at three months of determination using histological analysis in MT-treated male offspring. (F) ovary of fingerlings at three months of determination using histological analysis in MT-treated male offspring. (G) ovary of fingerlings at three months of determination using histological analysis in MT-treated neomale offspring. (H) ovary of fingerlings at three months of determination using histological analysis in MT-treated neomale offspring. The observed ovarian cell types included oogonia (OG) and previtellogenic oocytes (PVO). The observed testicular cell types include spermatogonia (SG), primary spermatocytes (PSC), secondary spermatocytes (SSC), and spermatids (ST). Scale bars represent 20 μm (C, E), 50 μm (A, D, F, G, H), and 100 μm (B).

4.4.4 Progeny testing of control males, MT-treated males, and MT-derived neomales using PACE genotyping

In addition, progeny was further subjected to PACE SNP assays to determine their genotypic sex. PACE genotyping results showed that progeny derived from control males and MT-treated males comprised both heterozygous male at the SNP_19 (G/T), SNP_20 (C/T), and SNP_21 (G/A) loci (Figure 4.6) and homozygous female at the SNP_19 (G/G), SNP_20 (C/C), and SNP_21(G/G) loci (Figure 4.6), whereas all progeny produced by MT-derived neomales exhibited a homozygous at the SNP_19 (G/G), SNP_20 (C/C), and SNP_21(G/G) loci (Figure 4.6). The genotypic results obtained from PACE assays were fully consistent with the phenotypic sex assignments determined by histological examination and squash method, and a summary of sex determination outcomes obtained using these methods is presented in (Table 4.3). In the control group, progeny derived from XY males crossed with XX females displayed a sex ratio close to 1:1, with female and male proportions of 50.83 ± 4.05% and 49.17 ± 4.05%, respectively. Statistical analysis revealed no significant deviation from the expected Mendelian ratio (χ^2 test, $P > 0.05$). Similarly, progeny from MT-treated XY males also exhibited an approximately equal sex ratio, with 49.45 ± 2.02% females and 50.56 ± 2.02% males, and no significant deviation from the 1:1 expectation was detected (χ^2 test, $P > 0.05$). In contrast, progeny produced by MT-derived neomales (XX genotype) crossed with XX females exhibited a strongly female-biased sex ratio,

with 100% female offspring observed. This sex ratio showed a highly significant deviation from the expected 1:1 ratio (χ^2 test, $P < 0.001$). Collectively, these results demonstrate that MT-derived neomales function as phenotypic males capable of successful fertilization, while transmitting a female (XX) genotype to all offspring, thereby enabling the production of an all-female progeny population.

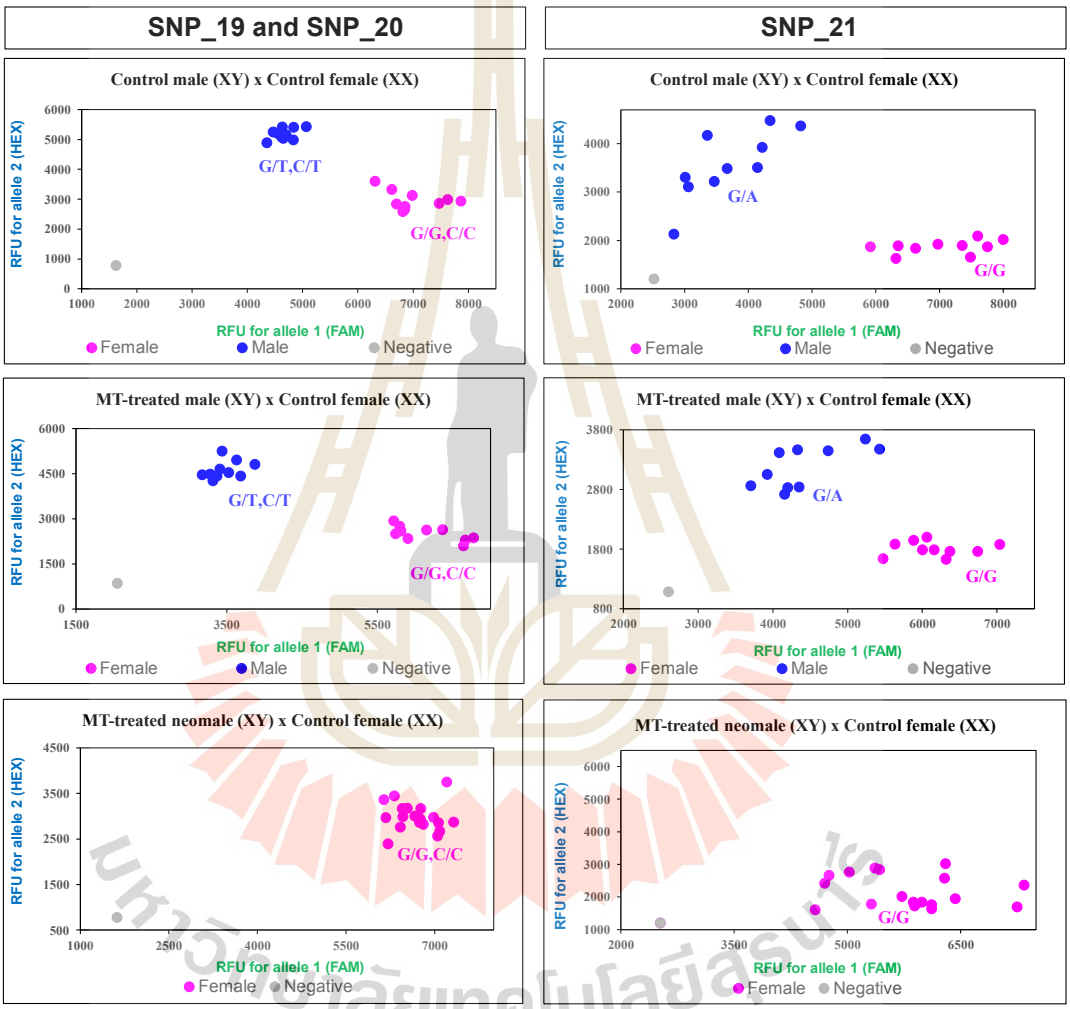

Figure 4.6 Segregation analysis of sex-specific SNPs (SNP_19, SNP_20, and SNP_21) in progeny using PACE assays. Scatter plots illustrate RFU signal intensities for allele 1 (FAM, x-axis) and allele 2 (HEX, y-axis). Each point represents an individual fish. Genotype distributions in progeny were consistent with the parental genotype patterns.

Table 4.3 Sex ratio and zootechnical characteristics of progeny produced from control females crossed with control males, MT-treated males, and MT-treated neomales.

	Body weight (g)	Total length (cm)	No. of males	No. of females	Male (%)	Female (%)	χ^2 *P*-value
Control male[1]							
C 1	7.13 ± 3.72	7.77 ± 1.24	29	31	48.33	51.67	0.7384
C 2	8.48 ± 5.12	8.15 ± 1.76	31	29	51.67	48.33	0.7384
C 3	10.53 ± 4.65	8.72 ± 1.08	33	27	55.00	45.00	0.3173
C 4	10.38 ± 5.30	9.03 ± 1.18	28	32	46.67	53.33	0.5054
C 5	10.28 ± 4.83	8.79 ± 1.47	26	34	43.33	56.67	0.1822
C 6	8.24 ± 9.87	7.58 ± 1.89	30	30	50.00	50.00	1.0000
Mean ± SD					*49.17 ± 4.05*	*50.83 ± 4.05*	
MT-treated male[1]							
M 1	4.74 ± 3.91	6.4 ± 1.62	32	28	53.33	46.67	0.5054
M 2	12.83 ± 5.32	9.34 ± 1.36	30	30	50.00	50.00	1.0000
M 3	7.75 ± 4.13	7.71 ± 1.25	31	29	51.67	48.33	0.7384
M 4	9.27 ± 4.34	8.08 ± 1.55	31	29	51.67	48.33	0.7384
M 5	10.22 ± 4.24	8.78 ± 1.12	29	31	48.33	51.67	0.7384
M 6	5.75 ± 3.15	7.10 ± 1.41	29	31	48.33	51.67	0.7384
Mean ± SD					*50.56 ± 2.02*	*49.45 ± 2.02*	
MT-treated neomale[1]							
N 1	11.00 ± 6.61	8.83 ± 1.54	0	60	0.00	100.00	<0.0000
N 2	13.27 ± 6.86	9.27 ± 1.72	0	60	0.00	100.00	<0.0000
N 3	13.18 ± 8.65	9.32 ± 1.76	0	60	0.00	100.00	<0.0000
N 4	10.94 ± 6.61	8.81 ± 1.54	0	60	0.00	100.00	<0.0000
N 5	12.97 ± 7.22	9.21 ± 1.80	0	60	0.00	100.00	<0.0000
N 6	13.17 ± 9.94	9.25 ± 1.97	0	60	0.00	100.00	<0.0000
Mean ± SD					*0.00 ± 0.00*	*100.00 ± 0.00*	

Abbreviations: C = control male; M = MT-treated male; N = MT-treated neomale. Genotypes of broodstock were determined using SNP_19, SNP_20, and SNP_21 (Figures 4.2–4.4). Gonadal sex of progeny was determined by squash method and histological analysis (Figure 4.5). Progeny genotypes were confirmed using PACE assays (Figure 4.6). Sex ratio deviations from the expected 1:1 ratio were analyzed using χ^2 tests. Values are presented as mean ± SD. All neomale-derived progeny showed significant deviation from 1:1 ($P < 0.001$).

4.5 Discussion

In the present study, sex-specific DNA markers enabled accurate identification of neomale genotypes, providing a reliable molecular tool for selective breeding. MT-derived neomales were successfully applied to generate an all-female progeny population in snakeskin gourami. Consistent with previous studies, indirect crosses between neomales and wild-type (WT) female broodstock have been widely used to produce all-female populations in teleost fishes, underscoring the effectiveness and broad applicability of neomale-based monosex breeding approaches (Luckenbach et al., 2017; Mu et al., 2024; Zhai et al., 2022). Notably, crosses involving MT-derived neomales consistently yielded exclusively female offspring, further validating the effectiveness of this neomale-based breeding strategy. These results provide direct evidence that MT-derived neomales function as phenotypic males, despite possessing a female (XX) genotype. Notably, MT-derived neomales were capable of normal spermatogenesis and successful fertilization, indicating that their reproductive performance is comparable to that of natural males. The production of exclusively female progeny further confirms the genetic female identity of neomales and demonstrates their effectiveness as functional broodstock for indirect, hormone-free monosex production.

4.5.1 MT-induced masculinization and molecular identification of neomales in snakeskin gourami

In teleost fishes, monosex populations have been widely produced through hormonal sex reversal, typically by administering sex hormones to sexually undifferentiated fry via the diet (Asad et al., 2024; El-Greisy and El-Gamal, 2012; Galbreath et al., 2003; Universidade Federal de Santa Catarina et al., 2018). Accordingly, the present study induced masculinization by feeding sexually undifferentiated fry a diet supplemented with the optimum dose of MT-100 diet. We produced MT-treated male fry and cultured them through the mature stage, providing MT-treated male broodstock. Note that control broodstock was also produced by feeding fry (7-97 dph) with a basal diet, which included both male and female broodstock. The resulting MT-treated fish exhibited male-like biological characteristics that persisted into adulthood, indicating effective and stable masculinization. Previous studies have reported that all-female production in snakeskin gourami can be achieved by hormonal treatment

during the early developmental period of 7–97 dph, and that estrogen-treated fish display growth performance comparable to genetic females, which show superior growth relative to males at adulthood (Kabpha et al., 2023). Building on these findings, the present study successfully produced a monosex male population using an optimized MT dosage, providing suitable broodstock for subsequent neomale-based breeding.

In our previous work, sex-specific SNP markers were developed to distinguish homozygous females from heterozygous males, with females exhibiting SNP_19 (G/G), SNP_20 (C/C), and SNP_21 (G/G) genotypes and males displaying SNP_19 (G/T), SNP_20 (C/T), and SNP_21 (G/A) genotypes (Chapter III). Using these markers in combination with Sanger sequencing and PACE SNP assays, the present study reliably identified the genotypic sex of broodstock, including females, control males, MT-treated males (XY), and MT-derived neomales (XX). Genotyping results demonstrated that MT-derived neomales shared identical genotypes with natural females, whereas both control males and MT-treated males exhibited heterozygous genotypes across all three loci. Consistent with reports in other fish species, such as rainbow trout and common carp (*Cyprinus carpio*), the application of molecular sex markers to identify neomales provides a powerful strategy for producing all-female populations through indirect breeding approaches (Colihueque et al., 2024; Xie et al., 2025).

4.5.2 Neomale-based all-female production and reproductive performance of MT-treated broodstock

Notably, MT-derived neomales were capable of generating exclusively female progeny, providing an effective indirect strategy for all-female production in snakeskin gourami. Accordingly, the present study employed a neomale-based breeding approach to achieve all-female progeny without the direct application of sex hormones during the grow-out phase. In fish species with an XX/XY sex determination system, hormonally induced neomale broodstock have been widely applied to generate all-female populations, thereby offering a practical means to eliminate the need for hormonal sex reversal in commercial production (Mu et al., 2024). The use of neomales as functional male broodstock for all-female production has also been successfully demonstrated in other species, including large yellow croaker and sablefish, highlighting the broader applicability of this strategy (Luckenbach et al., 2017;

Mu et al., 2024). Collectively, these findings establish a biotechnological framework for sustainable, hormone-free monosex aquaculture, with significant potential for future large-scale application.

According to previous reports, MT-treated broodstock in XX/XY sex determination systems may comprise both genetic males (XY) and sex-reversed neomales (Liu et al., 2021). In our study, progeny testing was performed to confirm male and neomale broodstock. Our results showed that 75% of the control males could produce offspring, while only 32.5% of the MT-treated males were able to produce offspring. These findings demonstrated that MT-treated males faced a reduction in reproductive performance. Therefore, further research and development are required to improve the reproductive performance of MT-treated male broodstock.

4.5.3 Progeny testing confirms the XX/XY sex determination system and the effectiveness of neomale-based all-female production

For progeny testing, histology of gonadal tissue and PACE SNP genotyping were applied to identify both phenotypic sex and genotypic sex, respectively. Our results showed that control male (XY × XX) mating and MT-treated male (XY × XX) mating produced the expected Mendelian 1:1 sex ratio, reaffirming the XX/XY sex determination system in snakeskin gourami. Expectedly, offspring derived from neomales (XX × XX) mating were uniformly female. The successful generation of all-female progeny through crosses between XX neomales and XX females confirms that MT-induced masculinization effectively produced functional male gametes, while maintaining a genetically female (XX) genotype in the offspring. These findings are consistent with previous reports in other teleost species, such as yellow croaker, common carp (Cyprinus carpio), and yellow drum (Nibea albiflora), in which neomale-based breeding strategies have also been successfully applied to generate all-female populations (Mu et al., 2024; Xie et al., Xu et al., 2018; 2026; Zhai et al., 2022).

Therefore, a combination of sex-specific DNA markers and MT-sex reversed male production could offer the effectiveness of neomale production technology in snakeskin gourami. This biotechnological approach provides a practical, hormone-free strategy for producing all-female populations. Future research is required to develop this technology at a farm scale for the commercialization of all-female snakeskin gourami farming.

4.6 Conclusion

In the present study, MT-treated male broodstock retained the ability to produce functional male gametes and successfully fertilize eggs, despite exhibiting reduced reproductive performance. Using sex-specific SNP markers, MT-derived neomales carrying a genetically female (XX) genotype were accurately identified and distinguished from genetic males. Subsequent crosses between XX neomales and XX females consistently produced all-female progeny, confirming both the functional male phenotype of neomales and the genetic basis of all-female inheritance. Compared with direct hormone application during early development, the neomale-based approach enables hormone-free production of all-female offspring, offering clear advantages in terms of food safety, regulatory acceptance, and sustainability. Overall, this study establishes an integrated biotechnological framework that combines hormonal sex reversal, molecular marker-assisted genotyping, and controlled breeding. Importantly, the consistent genotypic patterns and inheritance outcomes observed in progeny testing collectively provide strong evidence that snakeskin gourami possesses an XX/XY sex determination system, thereby supporting the practical application of neomale-based strategies for all-female production in this and other commercially important teleost species.

4.7 Acknowledgements

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CHAPTER V

Evidence for an XX/XY Sex Determination System in Snakeskin Gourami (*Trichopodus Pectoralis*) Revealed by 17β-Estradiol-Induced Neofemale

5.1 Abstract

In snakeskin gourami (*Trichopodus pectoralis*), females exhibit superior growth performance compared with males, highlighting the economic potential of all-female stocks in aquaculture. However, despite previous indications, additional genetic and functional evidence is still required to conclusively define the underlying sex determination system in this species. This study aimed to functionally validate neofemale- and supermale-based breeding strategies in snakeskin gourami through integrated progeny testing and sex-specific DNA marker analysis. in snakeskin gourami through integrated progeny testing and sex-specific DNA marker analysis. Neofemales were produced by dietary administration of 17β-estradiol (E2), and their genetic sex was confirmed using sex-linked SNP markers (SNP_19, SNP_20, and SNP_21). Progeny derived from control females, E2-treated females, and E2-treated neofemales were evaluated using a combination of squash methods, histological examination, and sex ratio analysis. Control and E2-treated females produced offspring with balanced 1:1 sex ratios, whereas crosses involving neofemales (XY genotype) resulted in a male-biased progeny ratio consistent with Mendelian expectations under an XX/XY system, confirming that neofemales function as fertile phenotypic females while retaining a male genetic constitution. Offspring from neofemales were further reared to identify supermales, which were confirmed by SNP genotyping and progeny testing. Crosses between supermales and wild-type females produced exclusively male offspring, demonstrating that supermales are fully fertile and transmit Y-linked sex-determining factors to all progenies. Notably, gonadal sex identified by the squash method showed complete concordance with histological observations at three months post-hatching, indicating that this approach provides a rapid, low-cost, and reliable method for large-

scale progeny sex identification. Collectively, the integration of molecular markers, progeny testing, and efficient phenotypic screening offers a practical framework for controlled monosex production and provides robust functional evidence supporting an XX/XY sex determination system in snakeskin gourami.

Keywords: Snakeskin gourami (*Trichopodus pectoralis*); Sex determination; Neofemale and supermale; Sex-specific SNP markers; Monosex production.

5.2 Introduction

Sex determination mechanisms in teleosts are remarkably diverse, ranging from classical male-heterogametic (XY) and female-heterogametic (ZW) systems to more complex arrangements such as X_1X_2Y, ZW_1W_2, and X0/XX (Fang et al., 2025). The identification of these systems has relied on a variety of cytogenetic, molecular, or genomic approaches, including karyotyping, fluorescent in situ hybridization (FISH), microsatellite-based linkage mapping, high-density SNP genotyping, and high-throughput sequencing technologies (e.g., RAD-seq, GWAS, and whole-genome assembly) (Sember et al., 2021; Whyte et al., 2007; Woram et al., 2003; Yang et al., 2024). Sex determination and sex control strategies represent critical components in modern aquaculture, particularly for species exhibiting pronounced sexual dimorphism in growth performance, physiology, or economic value, for instance, snakeskin gourami, half-smooth tongue sole (*Cynoglossus semilaevis*), largemouth bass (*Micropterus salmoides*), mandarin fish (*Siniperca chuatsi*), and sablefish (*Anoplopoma fimbria*), females generally grow faster, making them the favored sex for commercial culture (Bi et al., 2020; Kabpha et al., 2023; Luckenbach et al., 2017; Zhou and Chen, 2016). In contrast, species such as Northern snakehead (*Channa argus*), zig-zag eel (*Mastacembelus armatus*), and Nile tilapia (*Oreochromis* spp.) exhibit male-biased growth, which renders males more desirable for aquaculture purposes (Asad et al., 2024; Lu et al., 2024; Wang et al., 2019). In teleost fish, genetic sex determination systems are highly diverse and frequently interact with environmental or hormone factors, resulting in remarkable plasticity during early gonadal development (Devlin and Nagahama, 2002; Baroiller and D'Cotta, 2001). This plasticity has enabled the widespread application of hormonal sex reversal

techniques to manipulate phenotypic sex for monosex production, thereby improving production efficiency and stock uniformity in aquaculture systems (Pandian and Sheela, 1995; Piferrer, 2001).

In species with an XX/XY sex determination system, Direct monosex production using hormones has been successfully applied in species such as silver barb (*Puntius gonionotus* Bleeker), Medaka (*Oryzias latipes*), and black rockfish (*Sebastes schlegelii*) (Gaxheri et al., 2025; Scholz et al., 2003; Pongthana et al., 1999;). In particular, indirect monosex production based on sex-reversed broodstock of neomales or neofemales-derived supermales allow monosex populations to be generated without direct hormone exposure during the grow-out phase (Figure 5.1 A), like large yellow croaker (*Larimichthys crocea*), Common Carp (*Cyprinus carpio* L.), and mandarin fish (*Siniperca chuatsi*) (Liu et al., 2024; Mu, et al., 2024; Zhai et al., 2022). This approach addresses both regulatory and consumer concerns regarding hormone use in aquaculture. regarding hormone use in aquaculture (Penman and McAndrew, 2000; Devlin and Nagahama, 2002). However, the successful implementation of such strategies critically depends on two prerequisites: (i) confirmation that sex-reversed individuals are reproductively functional, and (ii) reliable identification of their underlying genetic sex. [Grab your reader's attention with a great quote from the document or use this space to emphasize a key point. To place this text box anywhere on the page, just drag it.]

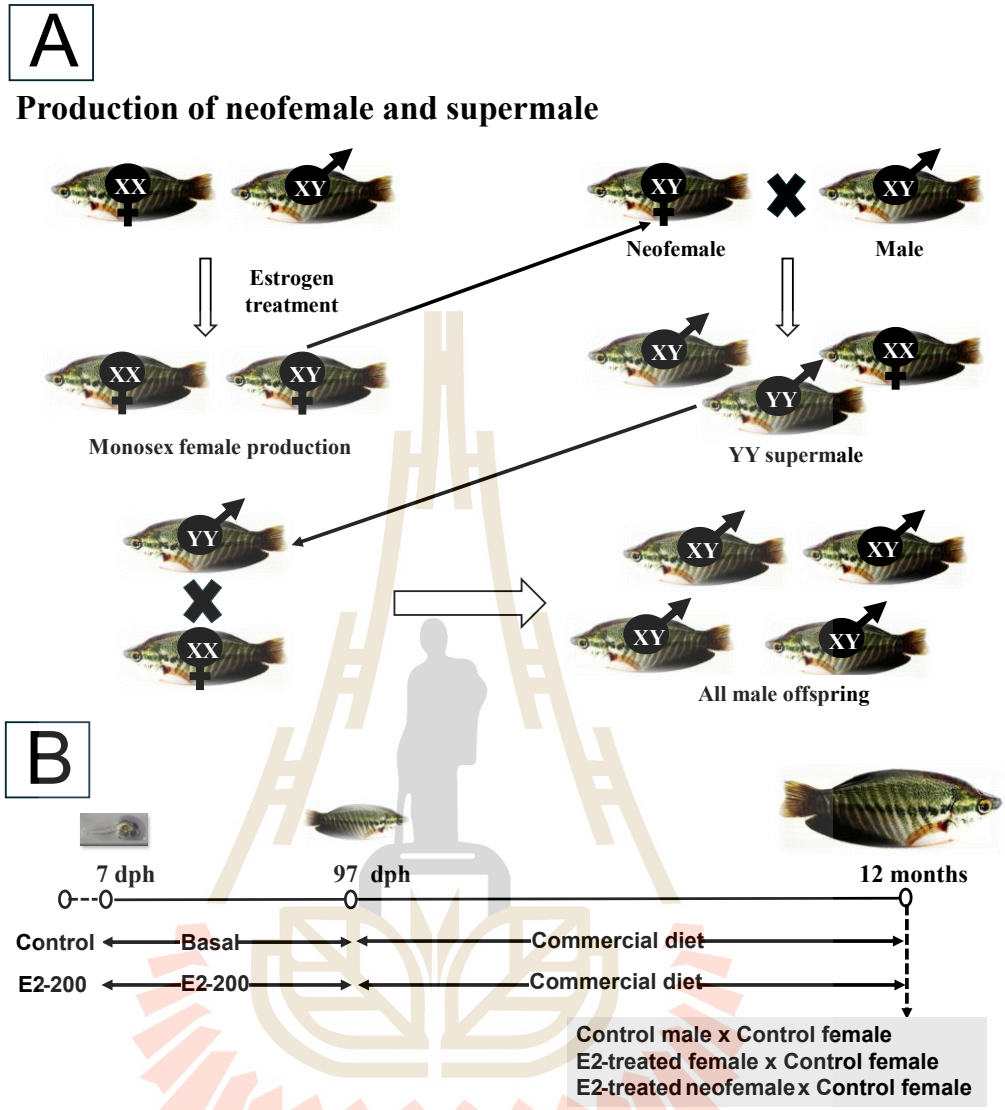

Figure 5.1 Experimental plan for E2-induced neofemale production in snakeskin gourami. **(A)** Schematic overview of sex reversal and breeding strategies under an XX/XY sex determination system, illustrating E2-induced neofemale production, supermale (YY) generation, and expected offspring sex outcomes. **(B)** Experimental workflow showing E2 treatment of 7-dph fry, broodstock development, controlled crosses, and progeny sex identification using sex-specific SNP markers (SNP_19, SNP_20, and SNP_21), squash method, and histological analysis.

Snakeskin gourami (*Trichopodus pectoralis*) is a commercially important freshwater species in Southeast Asia, where females exhibit superior growth performance compared with males (Panthum et al., 2021; Kabpha et al., 2023). Previous studies have demonstrated that dietary estrogen (E2) or androgen treatments can effectively induce sex reversal in this species, producing phenotypic females or males with growth and physiological characteristics comparable to those of natural counterparts (Kabpha et al., 2023). In a previous study, the physiological performance and gonadal development of hormonally sex-reversed broodstock were characterized, providing evidence that sex reversal does not impair reproductive potential (Chapter IV). Nevertheless, phenotypic observations alone are insufficient to distinguish true genetic females from sex-reversed individuals, particularly in broodstock intended for selective breeding.

Recent advances in molecular genetics have enabled the development of sex-specific DNA markers that allow unambiguous identification of genetic sex, independent of phenotypic appearance, such as Nile tilapia (*Oreochromis niloticus*), Spotted Knifejaw *(Oplegnathus punctatus), and* snakehead *(Channa maculata)* (Ma et al., 2022; Sun, ea al., 2014; Yang, et al., 2020) . In Chapter III, sex-linked SNP markers were developed for snakeskin gourami, enabling discrimination between homozygous females and heterozygous males and supporting an XX/XY sex determination system. While these markers provide a powerful tool for broodstock classification, their practical value must be validated through progeny testing to establish a direct link between parental genotype, reproductive performance, and offspring sex ratios.

Therefore, the primary objective of Chapter V was to integrate molecular sex identification with progeny testing to functionally validate neofemale and supermale broodstock in snakeskin gourami. Specifically, this chapter aims to (i) evaluate the reproductive performance of neofemales and supermales, (ii) assess gonadal development and sex differentiation in their offspring using squash method and histological approaches (Menu et al., 2005), and (iii) verify the consistency between offspring sex ratios and parental genotypes using sex-specific SNP markers. By combining phenotypic, histological, and molecular evidence, this chapter provides a comprehensive assessment of the feasibility of neofemale- and supermale-based

breeding strategies and delivers robust confirmation of an XX/XY sex determination system in snakeskin gourami.

5.3 Materials and methods

5.3.1 Ethics statement

All experimental procedures involving fish were reviewed and approved by the Animal Care and Use Committee of Suranaree University of Technology (approval no. 1731039).

5.3.2 Fish breeding and larval production

Sexually mature broodstock of snakeskin gourami (*Trichopodus pectoralis*) were obtained from commercial aquaculture farms located in Samut Sakhon Province, Thailand, and subsequently transported to the experimental facilities of the Suranaree University of Technology (SUT) farm in Nakhon Ratchasima, Thailand. Upon arrival, fish were acclimated to local rearing conditions prior to experimental use. Broodstock were maintained in a 0.04-ha earthen pond under ambient environmental conditions, including natural photoperiod and temperature fluctuations. Fish were fed a commercial formulated diet (Aquafeed, Thai Union Feedmill, Samut Sakhon, Thailand) containing 40% crude protein and 6% crude lipid. Feeding was conducted twice daily at 10:00 and 16:00 h to apparent satiation throughout the conditioning period. Artificial spawning was performed in five aerated concrete tanks (2 m × 2 m × 1 m), each filled with freshwater and equipped with floating aquatic vegetation to promote bubble nest formation, a characteristic reproductive behavior of this species. Each spawning tank was stocked with four males weighing 140–160 g and two females weighing 180–200 g, maintaining a male-to-female ratio of 2:1.

To induce ovulation, females were hormonally stimulated using a two-step intramuscular injection protocol. The initial injection consisted of 10 μg kg^{-1} body weight luteinizing hormone-releasing hormone analog (LHRHa; Suprefact, Hoechst, Germany) combined with 5 mg kg^{-1} domperidone (Motilium-M, OLIC, Bangkok, Thailand). A second injection containing 20 μg kg^{-1} LHRHa and 5 mg kg^{-1} domperidone was administered 12 h later. Males received a single intramuscular injection of LHRHa (10–20 μg kg^{-1} body weight) in combination with 5 mg kg^{-1} domperidone to

synchronize spermiation. Spawning activity was monitored regularly, and fertilized eggs were observed approximately 14 h after spawning. Following confirmation of fertilization, broodstock were promptly removed from the tanks to prevent egg predation. Fertilized eggs and newly hatched larvae were maintained in the same tanks under continuous aeration. Larvae were reared until 6 days post-hatching (dph), after which they were transferred into hapas for subsequent sex-reversal experiments.

5.3.3 Production of neofemales

Experimental diets were prepared by incorporating E2 (Sigma-Aldrich Chemical Co., St Louis, MO, USA) into a commercial diet (protein 50%, lipids 15%). The two treatment groups were as follows: basal diet (control), and 200 mg kg−1 (E-200) E2-supplemented diets. To prepare the hormonal treatment diets, E-200 was dissolved in 95% ethanol (240 mL) and sprayed over the commercial diet (1 kg). After evaporation of ethanol for 6 h in the dark at room temperature (26°C), the experimental diet was kept at 4°C until use.

Figure 5.1 B illustrates the experimental plan used in this study. For sex reversal experiment, the experimental units comprised 10 hapas (fine mesh aquatic dip net cages: 10 × 5 × 0.8 m) located in a cement pond (10 × 5 × 0.8 m3) with aeration and maintained at a 12:12 h light–dark cycle. Seven dph swim-up fry (n = 6 000) were transferred and randomly distributed to each hapa (600 fry/ hapa). The fish continued to grow in the same hapas and were fed basal diet and hormone-treated diets until 97 dph (Figure 5.1 B). The fish were fed four times daily (0900, 1130, 1400, and 1630) with experimental diets at 30% BW/day (month one), 20% BW/day (month two), and 15% BW/day (month three). A flow-through water-exchange system was used to replace one-third of the water weekly. Air and water temperatures were measured daily during the experimental period and were 23.0–33.0°C and 28.0–29.0°C, respectively. Throughout the sex reversal period, dissolved oxygen (DO) and pH were determined weekly using a DO and pH meter, respectively, and were within acceptable ranges of 4.50–6.83 mg L−1 and 7.75–8.10, respectively.

After three months of the sex reversal treatment (97 dph), the experimental fish were transferred to a cage for growth to become broodstock (Figure 5.1 B). Six cages; 4 × 8 × 1 m) were placed in two earthen ponds (0.08 ha) under a 12:12 h light–dark cycle. Each cage was released with 700 fish. Fish were hand-fed a

commercial diet (protein 28%, lipids 4%) twice daily ad libitum through the age of 12 months. Water was added to maintain a depth of 1 m. Air and water temperatures were measured daily during the experimental period and were 20–36°C and 28.3–29.9°C, respectively. DO and pH were determined weekly and were within acceptable ranges of 3.52–5.82 mg L^{-1} and 6.80–6.92, respectively.

5.3.4 Progeny testing of control group and E2-treated female group, and E2-treated neofemale group

Progeny testing was conducted in three mating groups including (i) control males crossed with wildtype (WT) females, (ii) E2-treated females paired with WT males, and (iii) E2-treated females paired with WT males. Control males (36 fish) were sampled for breeding with WT females using LHRHa and domperidone injection, and twenty-seven fish could produce offspring. Six male broodstock were sampled, and fin samples were collected for genotyping. Their offspring were cultured for sex determination. For E-200, fish (38 fish) were sampling for breeding with WT males using LHRHa and domperidone injection, and thirteen fish could produce offspring. The E2-treated fish which were able to produce offspring were sampling for fin collection for genotyping. 30 progeny populations were obtained from E2-treated females, and six progeny population were sampling for culture through 3 months for sex determination. The other six E2-treated females were neofemales, and their progeny were cultured for sex determination.

Fish breeding was conducted in mating tanks (2 m × 2 m × 1 m) with gentle aeration. Fourteen hours after spawning, the broodstock were removed from the tanks. Subsequently, larvae (7 dph) were fed a commercial diet (40% protein and 8% fat) until 30 dph. Thereafter, fingerlings from all groups were transferred to 2 × 2 × 2 m^3 hapas installed within a 0.08-ha earthen pond. Fish were hand-fed a commercial diet (35% protein and 4% lipid) through 3 months. At three months, individuals were randomly sampled for fin tissue collection, and gonads were collected for sex determination using squash method (Menu et al., 2005) and histological examination. Throughout the rearing period, air temperatures ranged from 22.0–35.0°C, while water temperatures were between 28.5–30.0°C.

5.3.5 Histological examination of the gonads

Sex determination was performed through histological analysis of gonadal tissue. Samples were fixed in Bouin's solution at 4 °C for 16 hours. Sex ratio

was determined in fish at 3 months of age (n = 60 fish per replicate; six replications). Fixed tissues were subjected to ethanol dehydration (70% to 100%), embedded in paraffin, and sectioned at 5 µm. The sections were mounted on glass slides, dewaxed, rehydrated with ethanol and xylene, and stained with hematoxylin and eosin. Gonadal morphology was then examined using a digital upright microscope (Olympus BX53, Tokyo, Japan).

5.3.6 Genotypic analysis using sex-specific SNP

Based on a previous study, sex-specific SNP markers, including SNP_19, SNP_20, and SNP_21, were developed to identify heterozygous males and homozygous females in snakeskin gourami (Chapter III). In this study, we used Sanger sequencing to determine the genotypic sex of control males, E2-treated females, E2-treated neofemales broodstock, and their offspring. Genomic DNA (gDNA) was extracted from fin tissue (approximately 100 mg) using the standard phenol-chloroform method. Briefly, fin samples were minced and incubated in lysis buffer containing proteinase K at 55°C until complete tissue digestion was achieved. Following lysis, an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1, v/v/v) was added, and the mixture was gently vortexed and centrifuged to separate the aqueous and organic phases. The upper aqueous phase containing DNA was carefully transferred to a new tube and further extracted with chloroform to remove residual phenol. gDNA was subsequently precipitated by the addition of cold absolute ethanol in the presence of sodium acetate, followed by centrifugation. The resulting DNA pellet was washed with 70% ethanol, air-dried, and resuspended in TE buffer. DNA concentration and purity were assessed using a NanoDrop™ ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) before downstream applications. Primer sequences, annealing temperatures, and expected amplicon sizes are presented in Table 5.1. PCR amplification was performed using TaKaRa Ex Taq polymerase (TaKaRa Bio, Shiga, Japan) in a total reaction volume of 25 µL. Each reaction mixture contained 2.5 µL 10× Ex Taq Buffer, 2 µL dNTP Mixture (2.5 mM each), 0.125 µL TaKaRa Ex Taq polymerase (5 U/µL), 2.5 µL forward primer (2 µM), 2.5 µL reverse primer (2 µM), 2.5 µL DNA template (50ng µL-1), and 12.875 µL nuclease-free water. PCR was performed under the following conditions: 95°C for 3 min; 35 cycles of 95°C for 30 s, annealing at the optimal temperature for each primer pair (55–65°C) for 30 s, and extension at 72°C for 25 s;

followed by a final extension at 72°C for 10 min. The target PCR products were excised from agarose gels and purified using the QIAEX® II Gel Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol for sequencing. Sanger sequencing was used to determine nucleotide variation at the target SNP locus.

5.3.7 Production of supermale

After progeny testing, the offspring from the neofemales were transferred to a new hapa for growth. 2 hapas (2 × 2 × 2 m^3) were placed in an earthen pond (0.08 ha) under a 12:12 hours light-dark cycle. Fish were hand -fed a commercial diet (protein 28%, lipids 4 %) twice per day ad libitum through the age of 12 months. Water was added to maintain a depth of 1 m. Air and water temperatures were measured daily during the experimental period and were 20 – 36°C and 28.3–29.9°C, respectively. DO, and pH were determined weekly and were within acceptable ranges of 3.52–5.82mgL-1 and 6.80–6.92, respectively.

5.3.8 Progeny testing of the control male group and supermale group

Progeny testing was performed using two mating groups: (i) control males crossed with wild-type (WT) females, and (ii) supermales crossed with WT females. In the control group, five breeding pairs were induced to spawn using LHRHa and domperidone injections. Fin clips were collected from all broodstock for genotyping before breeding, and the resulting offspring were reared until 3 months of age for sex determination. Similarly, five supermale broodstock were paired with WT females and induced to spawn using the same hormonal treatment. All five mating pairs successfully produced offspring. Fin samples were collected from the broodstock for genotyping, and five progeny populations were reared to 3 months post-hatching for subsequent sex determination.

Fish breeding was conducted in mating tanks (2 m × 2 m × 1 m) with gentle aeration. Fourteen hours after spawning, the broodstock were removed from the tanks. Subsequently, larvae (7 dph) were fed a commercial diet (40% protein and 8% fat) until 30 dph. Thereafter, fingerlings from all groups were transferred to 2 × 2 × 2 m^3 hapas installed within a 0.08-ha earthen pond. Fish were hand-fed commercial diet (35% protein and 4% lipid) for 3 months. At three months, individuals were randomly sampled for fin tissue collection, and gonads were collected for sex determination using the squash method (Menu et al., 2005) and histological

examination. Throughout the rearing period, air temperatures ranged from 22.0–35.0°C, while water temperatures were between 28.5–30.0°C.

5.3.9 Histological analysis of gonads

Sex identification was conducted by histological analysis of gonadal tissues. Samples were preserved in Bouin's fixative at 4°C for 16 h. The sex ratio was assessed in fish at three months of age (n = 60 individuals per replicate, with six replicates). After fixation, tissues were dehydrated using a graded ethanol series ranging from 70% to 100%, embedded in paraffin, and sectioned at a thickness of 5 µm. The sections were mounted on glass slides, followed by dewaxing and rehydration through xylene and ethanol gradients. Subsequently, the samples were stained with hematoxylin and eosin (H&E). Gonadal structures were observed under a digital upright microscope (Olympus BX53, Tokyo, Japan).

5.3.10 Genotypic analysis using sex-specific SNP

Similarly, the Sanger sequencing was used to determine the genotypic sex of control males, supermale broodstock, and their offspring (Chapter III). gDNA extraction was performed as described previously. Primer sequences, annealing temperatures, and expected amplicon sizes are presented in Table 5.1. PCR amplification and Sanger sequencing were performed as described previously.

Table 5.1 Sequences of primers used for Sanger sequencing.

Name	Primers (5′-3′)	Tm (°C)	Products (bp)	Application
SNP_19, 20	F: AATACGACTCACTATAGGGCCAGGGCTGTAACTCTGGACT R: GCACCTTGATCAAAGCACTCTCCA	63	551	Sanger sequencing
SNP_21	F: GAGACAGCTCTAGGGGCTCT R: TGCTCCTGTTTGGCCAGAAG	60	518	Sanger sequencing

Abbreviation: Tm (°C) = Annealing temperature (°C); bp= base pair.

Note: The underlined sequence AATACGACTCACTATAGGG represents the T7 universal sequencing tag added to the 5′end of primers.

5.3.11 Data analysis

All statistical analyses were performed using SPSS software version 22.0 (SPSS Inc., Chicago, IL, USA). Chi-square (χ^2) goodness-of-fit tests were applied to assess deviations from the expected 1:1 sex ratio in the E2-treated female, E2-treated neofemale, control, and supermale groups. In addition, chi-square tests were used to compare the sex ratios of progeny among the E2-treated female, E2-treated neofemale, control, and supermale groups. Differences were considered statistically significant at $P < 0.05$.

5.4 Results

5.4.1 Genotypic identification of control females, E2-treated females, E2-treated neofemales, and males broodstock by Sanger sequencing

Table 5.2 shows that, among the 36 selected mature control males, 27 individuals successfully produced offspring, corresponding to a mating success rate of 75.0%. Similarly, 30 out of 38 E2-treated individuals were fertile, yielding a mating success rate of 78.0%. For progeny testing, sixty offspring from each mating group—control males, E2-treated females, and E2-treated neofemales—were randomly selected and reared for 90 days, after which sex ratios were determined. The genotypes of all broodstock, including control females, E2-treated females, E2-treated neofemales, and males, were identified by PCR amplification followed by Sanger sequencing. Consistent with previous findings demonstrating an XX/XY sex determination system in snakeskin gourami (Chapter III), genotyping analysis revealed that control females and E2-treated females were homozygous at SNP_19 (G/G), SNP_20 (C/C), and SNP_21 (G/G; complementary C/C detected in antisense sequencing). In contrast, E2-treated neofemales and control males exhibited heterozygous genotypes at SNP_19 (G/T), SNP_20 (C/T), and SNP_21 (G/A; C/T detected in antisense sequencing) (Figure 5.2 and 5.3).

Table 5.2 Number of control male and E2-terated female broodstock that were used for progeny testing.

Parameters	Control group		E2-200 group	
	Males	females	females	Control males
No. of broodstock	36	36	38	38
No. of pairs with hatching	27	27	30	30
No. of pairs with no hatching	9	9	8	8
Mating success (%)	75.0	75.0	78.95	78.95

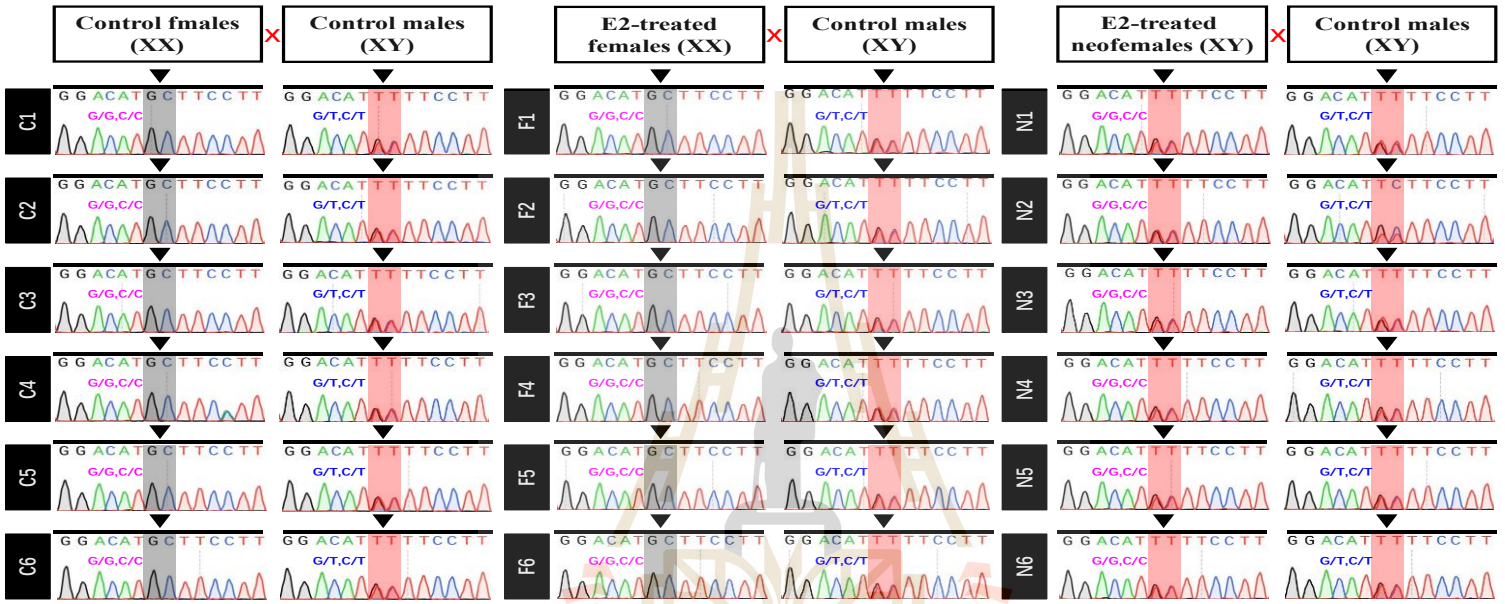

Figure 5.2 Identification of sexual genotypes at SNP_19 and SNP_20 in broodstock using Sanger sequencing. Representative chromatograms from control females (XX) × control males (XY) (C1–C6), E2-treated females (XX) × control males (XY) (F1–F6), and E2-treated neofemales (XY) × control males (XY) (N1–N6) showed that control females and E2-treated females were consistently homozygous at SNP_19 (G/G) and SNP_20 (C/C), whereas control males and E2-treated neofemales were heterozygous at both loci (SNP_19: G/T; SNP_20: C/T). These results confirm that E2-treated neofemales retained a male (XY) genetic constitution despite exhibiting a female phenotype. Abbreviations: C = control group; F = E2-treated female; N = E2-treated neofemale.

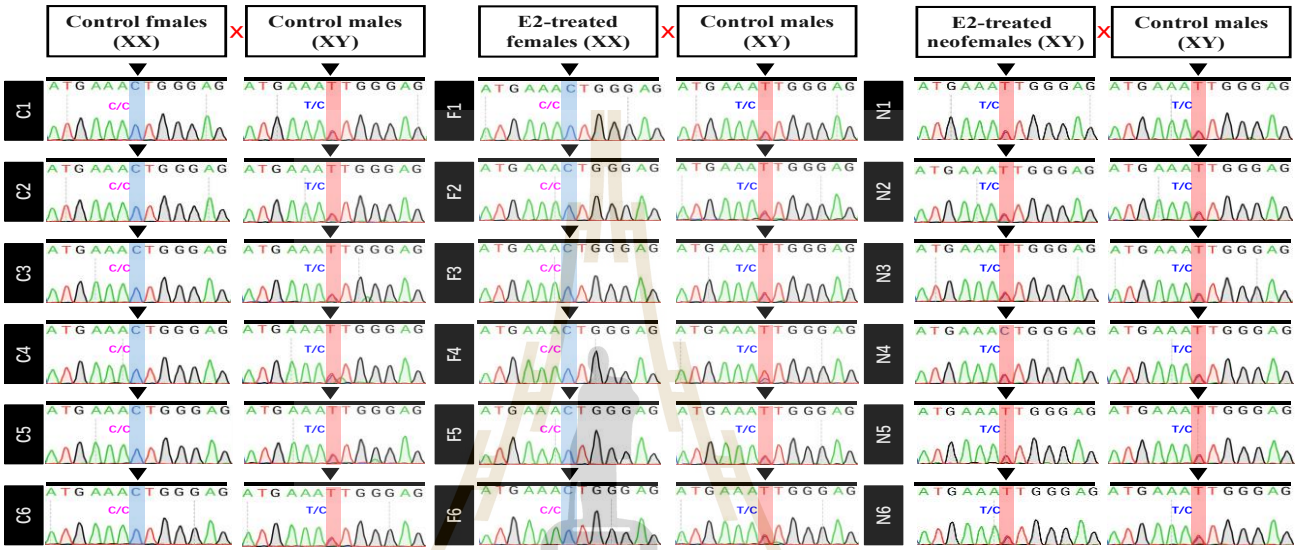

Figure 5.3 Identification of sexual genotypes at SNP_21 in broodstock using Sanger sequencing. Representative chromatograms from control females (XX) × control males (XY) (C1–C6), E2-treated females (XX) × control males (XY) (F1–F6), and E2-treated neofemales (XY) × control males (XY) (N1–N6) showed that control females and E2-treated females were consistently homozygous at SNP_21 (G/G; complementary C/C detected in antisense sequencing), whereas control males and E2-treated neofemales were heterozygous at SNP_21 (G/A; complementary C/T detected in antisense sequencing). These results confirm that E2-treated neofemales retained a male (XY) genetic constitution despite complete phenotypic feminization. Abbreviations: C = control group; F = E2-treated female; N = E2-treated neofemale.

5.4.2 Progeny testing of control females, E2-treated females, and E2-treated neofemales using the squash method and histological analysis

Gonadal sex of progeny was initially determined using the squash method based on cellular morphological characteristics. As illustrated in (Figure 5.4 A and B), ovarian and testicular tissues were readily distinguishable in female and male progeny, respectively. To verify the reliability of this approach, corresponding samples were further examined by histological analysis, which consistently confirmed ovarian structures in females and testicular structures in males. In female progeny, squash preparations revealed abundant round oocytes with clear cellular boundaries (Figure 5.4 A), closely matching the ovarian features observed in histological sections. Histological examination demonstrated ovaries composed primarily of oogonia (OG) and previtellogenic oocytes (PVO), indicative of an immature ovarian stage (Figure 5.4 C, E, and G). In contrast, squash preparations from male progeny showed compact clusters of spermatogenic cells, mainly spermatocytes (Figure 5.4 B). Consistently, histological sections revealed well-defined lobular testes containing spermatogonia (SG), primary spermatocytes (PSC), secondary spermatocytes (SSC), and a small number of spermatids (ST) distributed throughout the testicular tissue (Figure 5.4 D, F, and H). Comparable gonadal characteristics were observed among progeny derived from control males, E2-treated females, and E2-treated neofemales, all of which exhibited typical features of immature ovaries or testes at the juvenile stage (Figure 5.4 C–H). The high concordance between squash observations and histological findings indicates that gonadal sex in snakeskin gourami progeny can be reliably distinguished by distinct gonadal morphology at 3 months post-hatching. These results confirm that the squash method represents a rapid, accurate, and efficient approach for early sex identification and large-scale progeny screening.

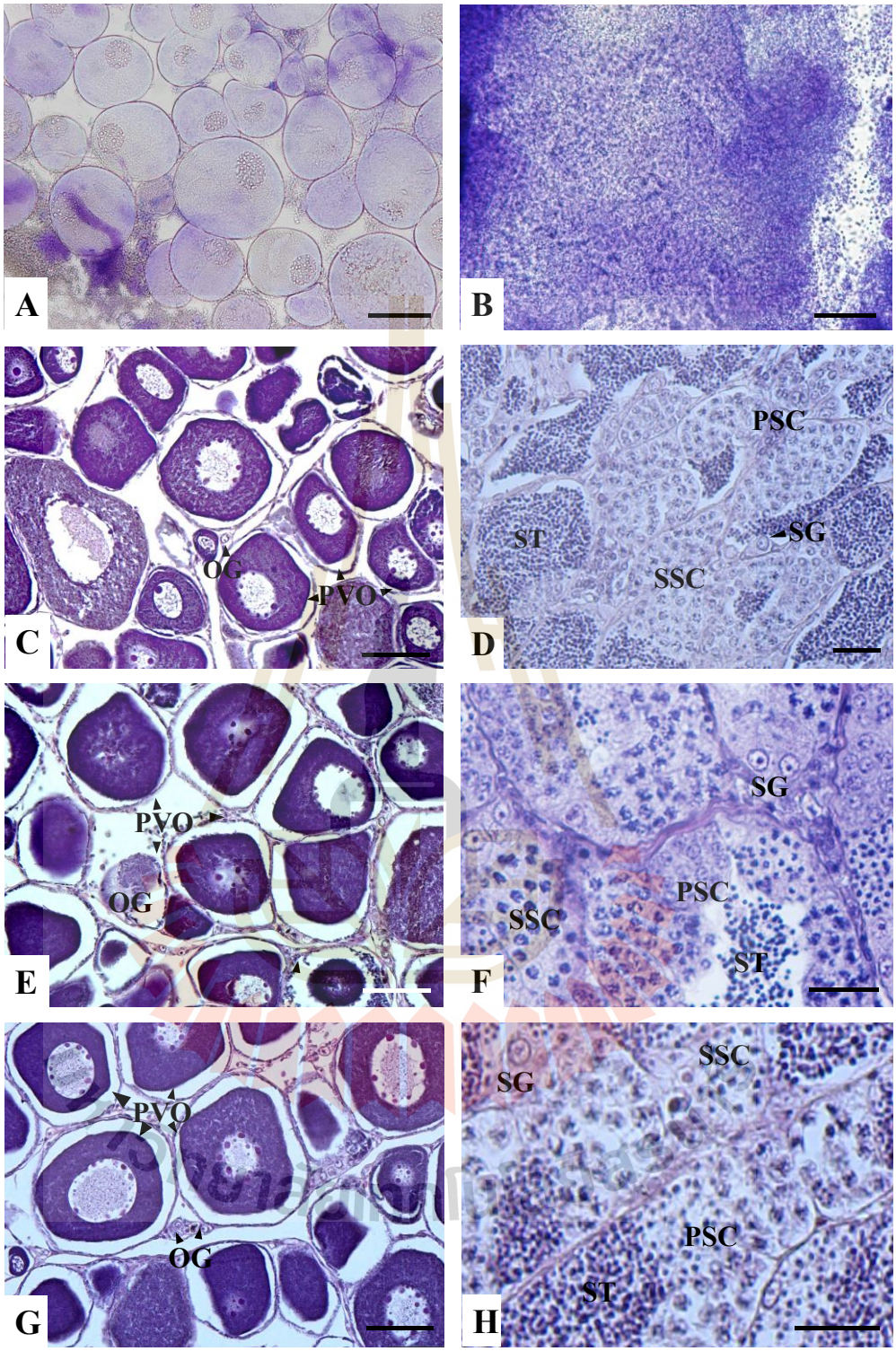

Figure 5.4 Histological and squash-based characterization of gonadal development in progeny derived from neofemale experiments in snakeskin gourami. (A) Ovarian tissue of progeny at three months was identified by the squash

method, showing numerous rounded oocytes. (B) Testicular tissue of progeny at three months was identified by the squash method, characterized by dense clusters of spermatogenic cells. (C) Histological section of the ovary from control group offspring at three months. (D) Histological section of testis from control group offspring at three months. (E) Histological section of the ovary from E2-treated female offspring at three months. (F) Histological section of testis from E2-treated female offspring at three months. (G) Histological section of the ovary from E2-treated neofemale offspring at three months. (H) Histological section of testis from E2-treated neofemale offspring at three months. The observed ovarian cell types included oogonia (OG) and previtellogenic oocytes (PVO). The observed testicular cell types include spermatogonia (SG), primary spermatocytes (PSC), secondary spermatocytes (SSC), and spermatids (ST). Scale bars represent 100 μm (A, B), 50 μm (C, E, G), and 20 μm (D, F, H).

A summary of sex determination results obtained using these methods is presented in (Table 5.3). In the control group, progeny produced by crosses between XY males and XX females displayed an approximately balanced sex ratio, with male and female proportions of 48.33 ± 3.94% and 51.67 ± 3.94%, respectively, showing no significant deviation from the expected Mendelian 1:1 ratio (χ^2 test, $P > 0.05$). Similarly, offspring derived from E2-treated XX females exhibited a comparable sex distribution, with 47.50 ± 5.03% males and 52.50 ± 5.03% females, and no significant departure from the expected ratio was detected (χ^2 test, $P > 0.05$). In contrast, progeny obtained from crosses between E2-treated neofemales (XY genotype) and XY males showed a pronounced male-biased sex ratio consistent with the expected 3:1 male-to-female ratio, with no significant deviation from this expectation (χ^2 test, $P > 0.05$). Taken together, these findings demonstrate that E2-treated neofemales function as fertile phenotypic females.

Table 5.3 Sex ratio and body measurements of progeny derived from control, E2-treated female, neofemale broodstock.

Group	progeny Body weight (g)	Total length (cm)	No. of males	No. of females	Male (%)	Female (%)	χ^2 *P*-value
Control female							
C 1	8.65 ± 4.99	8.24 ± 1.69	32	28	53.33	46.67	0.6056
C 2	6.86 ± 5.6	7.39 ± 1.93	28	32	46.67	53.33	0.6056
C 3	13.22 ± 5.23	9.47 ± 1.31	30	30	50.00	50.00	1.0000
C 4	5.45 ± 3.16	6.95 ± 1.43	27	33	45.00	55.00	0.4386
C 5	5.10 ± 2.58	7.06 ± 1.16	31	29	51.67	48.33	0.7963
C 6	15.82 ± 11.45	9.58 ± 2.17	26	34	43.33	56.67	0.3017
Mean ± SD					*48.33 ± 3.94*	*51.67 ± 3.94*	
ES-treated female							
F 1	8.95 ± 3.5	8.22 ± 0.89	24	36	40.00	60.00	0.1213
F 2	10.47 ± 5.95	8.64 ± 1.55	27	33	45.00	55.00	0.4386
F 3	19.47 ± 8.27	10.84 ± 1.61	30	30	50.00	50.00	1.0000
F 4	12.23 ± 8.39	8.69 ± 2.01	33	27	55.00	45.00	0.4386
F 5	10.8 ± 3.84	8.83 ± 1.06	28	32	46.67	53.33	0.6056
F 6	6.85 ± 2.81	7.7 ± 1.02	29	31	48.33	51.67	0.7963
Mean ± SD					*47.50 ± 5.03*	*52.50 ± 5.03*	
E2-treated neofemale							
N 1	13.02 ± 6.04	9.21 ± 1.48	46	14	76.67	23.33	0.7656
N 2	8.88 ± 3.8	8.49 ± 1.14	46	14	76.67	23.33	0.7656
N 3	11.62 ± 6.58	8.96 ± 1.7	45	15	75.00	25.00	1.0000
N 4	8.63 ± 3.8	8.29 ± 0.95	42	18	70.00	30.00	0.3711
N 5	8.12 ± 3.85	8.14 ± 1.4	42	18	70.00	30.00	0.3711
N 6	12.45 ± 7.32	9.01 ± 2.06	43	17	71.67	28.33	0.5510
Mean ± SD					*73.34 ± 3.16*	*26.67 ± 3.16*	

Abbreviations: C = Control group; F = ES-treated female; N = ES-treated neofemale

Note: Values are expressed as mean ± SD. The χ^2 test was used to assess deviations from the expected 1:1 sex ratio in the control group. Statistical significance was set at $p < 0.05$.

5.4.3 Reproductive performance and genotypic identification of control males and supermales

To evaluate reproductive capacity, control males and YY supermales were subjected to progeny testing. As summarized in Table 5.4, control males showed

a mating success rate of 75.0%, with 27 out of 36 pairs successfully producing hatched larvae. In contrast, all supermales successfully produced offspring, achieving a 100% mating success rate. These results indicate that YY supermales retained normal fertility. Genotypic identification of broodstock was subsequently performed by PCR amplification followed by Sanger sequencing. Consistent with the established XX/XY sex determination system in snakeskin gourami (Chapter III), control males exhibited heterozygous genotypes at SNP_19 (G/T), SNP_20 (C/T), and SNP_21 (G/A). In contrast, supermales were homozygous at these loci, displaying SNP_19 (T/T), SNP_20 (T/T), and SNP_21 (A/A). All control females were homozygous at SNP_19 (G/G), SNP_20 (C/C), and SNP_21 (G/G) (Figures 5.5 and 5.6). These results confirm the successful production of YY supermales and validate the reliability of the identified sex-specific SNP markers.

Table 5.4 Number of control male broodstock and neofemale-derived supermale broodstock used in progeny testing.

Parameters	Control group		Supermale group	
	Males	females	Supermale	Control females
No. of broodstock	36	36	5	5
No. of pairs with hatching	27	27	5	5
No. of pairs with no hatching	9	9	0	0
Mating success (%)	75.0	75.0	100	100

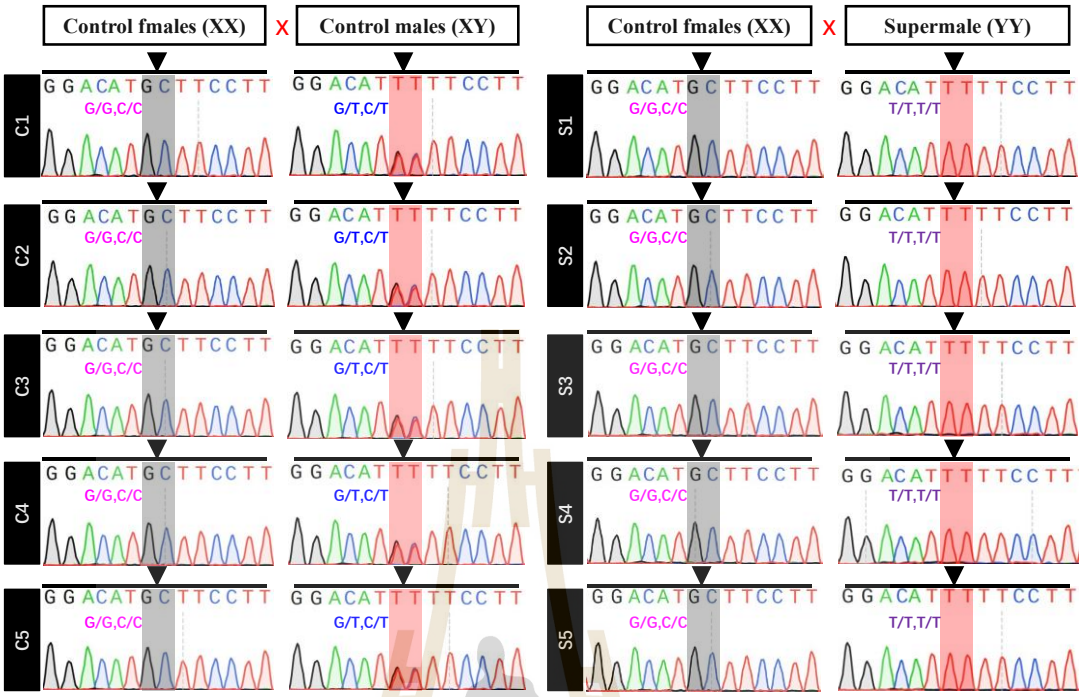

Figure 5.5 Identification of control males and supermales based on sex-specific SNP markers (SNP_19 and SNP_20) using Sanger sequencing Representative chromatograms from control females (XX) × control males (XY) (C1–C5) and control females (XX) × supermales (YY) (S1–S5) showed that control males were consistently heterozygous at SNP_19 (G/T) and SNP_20 (C/T), whereas supermales were homozygous at both loci (SNP_19: T/T; SNP_20: T/T). In contrast, control females were homozygous at SNP_19 (G/G) and SNP_20 (C/C). These results confirm that supermales retained a homozygous genotype pattern distinct from control males, validating the successful production of YY supermales.

Abbreviations: C = control group; S = supermale group.

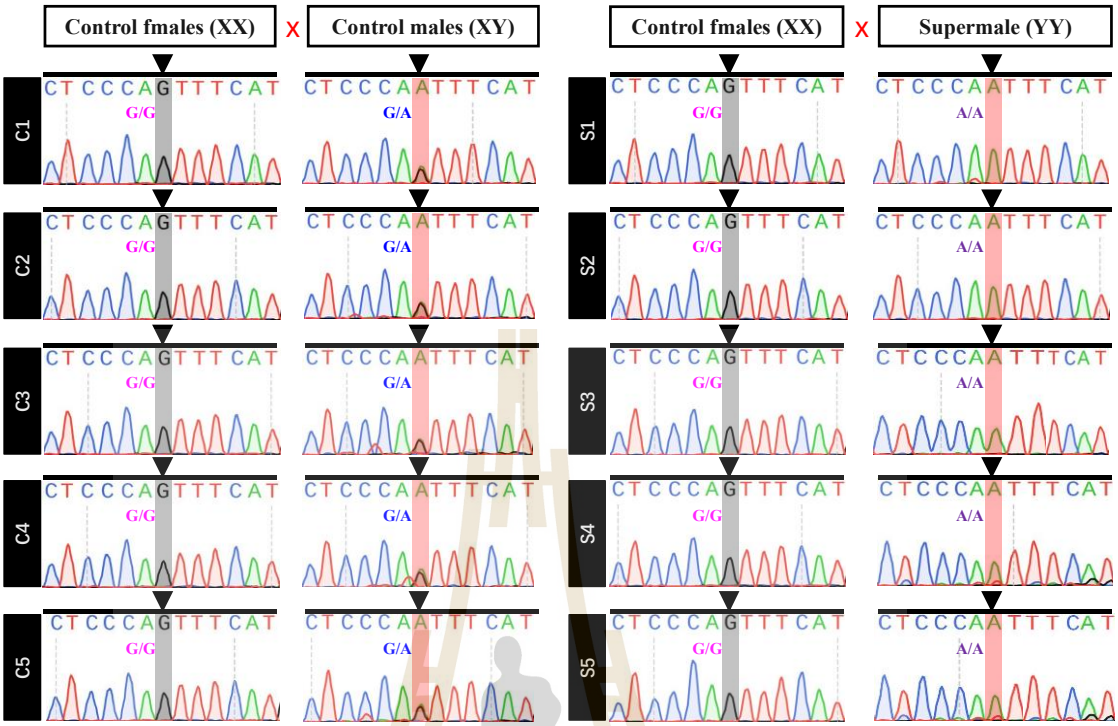

Figure 5.6 Identification of control male and supermale genotypes using the sex-specific SNP marker SNP_21 by Sanger sequencing. Representative chromatograms from control females (XX) × control males (XY) (C1–C5) and control females (XX) × supermales (YY) (S1–S5) showed that control males were consistently heterozygous at SNP_21 (G/A), whereas supermales were homozygous at SNP_21 (A/A). In contrast, control females were homozygous at SNP_21 (G/G). These results confirm that supermales retained a homozygous genotype pattern distinct from control males, providing further molecular validation of successful YY supermale production.

Abbreviations: C = control group; S = supermale group.

5.4.4 Progeny testing of control males and supermale using the squash method and histological analysis

Gonadal sex of progeny was initially assessed using the squash method based on cellular morphology. As shown in (Figure 5.7 A and B), ovarian and testicular tissues were clearly distinguishable in female and male progeny, respectively. To evaluate the reliability of this method, representative samples were further subjected

to histological examination, which consistently confirmed ovarian structures in females and testicular structures in males. In female progeny, squash preparations revealed numerous rounded oocytes with well-defined cellular boundaries (Figure 5.7 A), corresponding closely to ovarian morphology observed in histological sections. Histological analysis showed ovaries primarily composed of oogonia (OG) and previtellogenic oocytes (PVO), indicating an immature ovarian stage (Figure 5.7 C). In contrast, squash preparations from male progeny displayed dense clusters of spermatogenic cells, predominantly spermatocytes (Figure 5.7 B). Consistently, histological sections revealed well-organized lobular testes containing SG, PSC, SSC, and a small number of ST distributed throughout the testicular tissue (Figure 5.7 D-F). Similar gonadal features were observed in progeny derived from control males (Figure 5 .7 C and D), and supermales (Figure 5.7 E and F), all of which exhibited typical characteristics of immature testes at the juvenile stage. The strong agreement between squash observations and histological findings indicates that gonadal sex in snakeskin gourami progeny can be reliably identified based on gonadal morphology at 3 months post-hatching.

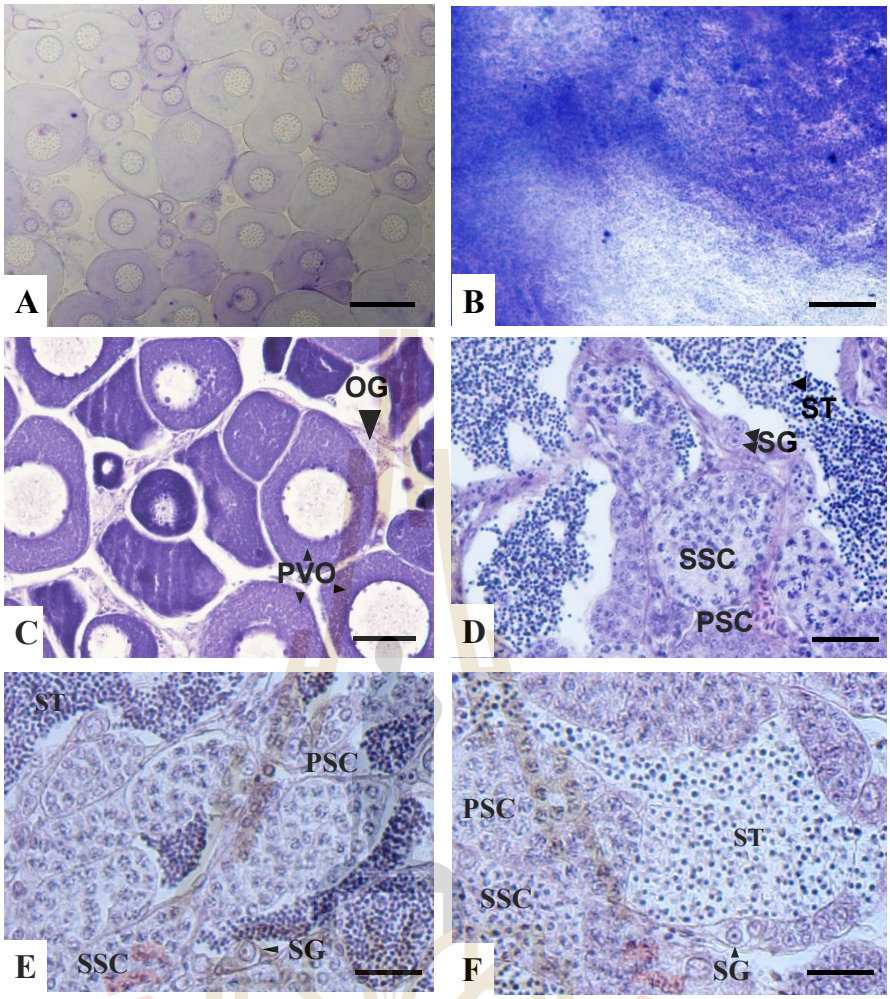

Figure 5.7 Histological and squash-based characterization of gonadal development in progeny derived from supermale experiments in snakeskin gourami. (A) Squash preparation of the ovary from progeny at three months, showing numerous rounded oocytes with clear cellular boundaries. (B) Squash preparation of testis from progeny at three months, characterized by dense clusters of spermatogenic cells. (C) Histological section of ovary from progeny derived from control males at three months. (D) Histological section of testis from progeny derived from control males at three months. (E and F) Histological section of testis from progeny derived from supermales at three months. The observed ovarian cell types included OG and PVO. The observed testicular cell types include SG, PSC, SSC, and ST. Scale bars represent 100 μm (A, B), 50 μm (C), and 20 μm (E, F).

A summary of sex determination results obtained using these approaches is presented in (Table 5.5). In the control group, progeny produced from crosses between XY males and XX females exhibited an approximately balanced sex ratio, with 51.33 ± 5.05% males and 48.67 ± 5.05% females, showing no significant deviation from the expected Mendelian 1:1 ratio (χ^2 test, $P > 0.05$). In contrast, offspring derived from supermale (YY) males displayed an exclusively male phenotype, with 100.00 ± 0.00% males and no females detected, representing a highly significant deviation from the 1:1 expectation (χ^2 test, $P < 0.001$). Taken together, these results demonstrate that supermales function as fertile phenotypic males and provide further confirmation that sex determination in snakeskin gourami follows an XX/XY genetic system.

Table 5.5 Sex ratio and body measurements of progeny derived from control, supermale broodstock.

Group	progeny Body weight (g)	Total length (cm)	No. of males	No. of females	Male (%)	Female (%)	χ^2 *P*-value
Control							
C 1	7.13±3.72	7.77±1.24	29	31	48.33	51.67	0.7963
C 2	9.4±3.22	8.62±1.12	35	25	58.33	41.67	0.1976
C 3	10.53±4.65	8.72±1.08	33	27	55.00	45.00	0.4386
C 4	10.38±5.3	9.03±1.18	28	32	46.67	53.33	0.6056
C 5	11.5±5.59	9.16±1.4	29	31	48.33	51.67	0.7963
					51.33 ± 5.05	*48.67 ± 5.05*	
Supermale							
S1	6.38 ± 4.06	7.26 ± 1.4	60	0	100.00	0.00	<0.0000
S2	12.2 ± 5.92	9.15 ± 1.57	60	0	100.00	0.00	<0.0000
S3	8.8 ± 4.3	8.44 ± 0.98	60	0	100.00	0.00	<0.0000
S4	8.38 ± 2.43	8.47 ± 0.75	60	0	100.00	0.00	<0.0000
S5	8.06 ± 3.76	8.13 ± 1.36	60	0	100.00	0.00	<0.0000
Mean ± SD					*100.00 ± 0.00*	*0.00 ± 0.00*	

Abbreviations: C = Control group; S = Supermale group.

Note: Values are expressed as mean ± SD. The χ^2 test was used to assess deviations from the expected 1:1 sex ratio in the control group and supermales.

Statistical significance was set at $p < 0.05$.

5.5 Discussion

Sex determination and control are key research areas in aquaculture biology, particularly for economically significant species that show sexual dimorphism in growth, survival, or market value. In snakeskin gourami (*Trichopodus pectoralis*), previous studies have suggested a genetic XX/XY sex determination system (Panthum et al., 2021); however, the lack of stable sex-specific markers and validated breeding strategies has limited the practical application of sex control in this species. The present study integrates endocrine manipulation, progeny testing, gonadal histology, and sex-specific SNP genotyping to establish a complete and verifiable pathway to produce functional neofemales and supermales. By combining phenotypic, histological, and molecular evidence, this work provides a closed-loop validation of genetic sex determination and demonstrates a feasible strategy for controlled monosex production in snakeskin gourami.

5.5.1 Molecular validation of estrogen-induced sex reversal using sex-specific SNP

A previous study reported that dietary administration of 200 mg kg^{-1} E2 successfully produced an all-female population of snakeskin gourami (Kabpha et al., 2023). In the present study, the same E2 dosage was adopted to induce feminization and generate an all-female population of this species. One of the primary objectives of this study was to evaluate whether dietary E2 administration could effectively induce sex reversal while maintaining normal gametogenesis and fertilization capacity in treated broodstock. The observed mating success rate of E2-treated broodstock (78.0%) was comparable to that of untreated control males (75.0%), indicating that estrogen exposure during early developmental stages did not adversely affect subsequent gonadal maturation or fertility. This finding is consistent with previous studies in teleost fish demonstrating that estrogen-induced sex reversal, when applied within a critical developmental window, can redirect gonadal differentiation while preserving reproductive function (Pandian and Sheela, 1995; Devlin and Nagahama, 2002; Piferrer, 2001).

In our previous study, sex-specific SNP markers were developed to distinguish homozygous females from heterozygous males, with females exhibiting

SNP_19 (G/G), SNP_20 (C/C), and SNP_21 (G/G) genotypes and males displaying SNP_19 (G/T), SNP_20 (C/T), and SNP_21 (G/A) genotypes (Chapter III). In this study, control females and E2-treated females consistently exhibited homozygosity at all three sex-specific loci (SNP_19, SNP_20, and SNP_21), whereas E2-treated neofemales and males displayed heterozygous genotypes at the same loci. This clear and reproducible genotype–phenotype correspondence strongly supports the presence of an XX/XY sex determination system in this species and demonstrates the reliability of these SNP markers for distinguishing genetic sex independent of phenotypic appearance. Importantly, the identification of heterozygous genotypes in E2-treated neofemales provides unambiguous molecular confirmation that these individuals are genetic males (XY) that have undergone complete phenotypic feminization. This distinction is particularly critical, as phenotypic observations alone cannot reliably differentiate between true genetic females and sex-reversed individuals. The application of sex-specific SNP markers, therefore, eliminates ambiguity in broodstock classification and ensures accurate interpretation of progeny testing results. Similar marker-assisted approaches have been widely adopted in other teleost species to validate sex reversal and to avoid misclassification caused by environmental or hormonal influences on gonadal development (Huang et al., 2024; Ou et al., 2025; Penman et al., 2008; Yang et al., 2024). The consistency of SNP genotypes across control males, E2-treated neofemales, and phenotypic females further indicates that estrogen treatment altered gonadal differentiation without modifying the underlying genetic sex. the SNP markers demonstrated clear diagnostic reliability and are compatible with routine PCR and Sanger sequencing workflows, making them practical tools for broodstock screening and genetic management in aquaculture operations.

5.5.2 Functional validation of E2-induced neofemales through phenotypic and progeny sex ratio analyses

The combined application of the squash method and histological analysis provided a robust framework for phenotypic sex identification in juvenile snakeskin gourami progeny. This approach has previously been applied successfully for sex identification in juvenile fishes, demonstrating its broad applicability across teleost species (Cungihan et al., 2024; Menu et al., 2005). The clear correspondence between cellular morphology observed in squash preparations and gonadal structures revealed

by histological sections indicates that gonadal differentiation at three months post-hatching is sufficiently advanced to allow reliable sex discrimination, consistent with typical patterns of juvenile gonadal development reported in teleost fishes. The strong concordance between these two independent methods confirms that the squash technique represents a rapid, accurate, and practical tool for early sex determination, particularly well-suited for large-scale progeny screening in breeding programs. Importantly, comparable gonadal morphologies were observed among progeny derived from control males, E2-treated females, and E2-treated neofemales, indicating that parental E2 treatment did not induce abnormal gonadal development in the offspring. This finding suggests that estrogen-induced sex reversal in broodstock does not exert transgenerational effects on gonadal differentiation, which is similar to the common snook (*Centropomus undecimalis*) (Contreras-García et al., 2023).

Progeny sex ratio analyses provided additional genetic insight into the functional role of E2-treated neofemales. As expected under an XX/XY sex determination system, crosses between control XY males and XX females yielded an approximately balanced 1:1 sex ratio, confirming normal Mendelian segregation. Similarly, progeny produced by E2-treated XX females also exhibited sex ratios indistinguishable from the control group, indicating that estrogen treatment did not alter the genetic sex composition of these females. In contrast, crosses involving E2-treated neofemales (XY genotype) and XY males resulted in a pronounced male-biased sex ratio consistent with the theoretical 3:1 male-to-female expectation. The absence of significant deviation from this ratio provides strong genetic evidence that neofemales produce functional gametes carrying either X or Y chromosomes.

Collectively, the concordant phenotypic and sex ratio data demonstrate that E2-treated neofemales function as fully fertile phenotypic females while retaining a male (XY) genetic constitution. This dual validation—phenotypic sex identification supported by progeny testing—provides a critical link between molecular genotyping and reproductive performance. The ability of neofemales to generate predictable sex ratios in their offspring establishes a solid foundation for subsequent supermale (YY) production and confirms the feasibility of applying controlled sex reversal strategies in snakeskin gourami aquaculture.

5.5.3 Validation of sex-specific SNP markers for genetic sex identification in broodstock

Accurate identification of genetic sex is a fundamental prerequisite for the establishment of monosex breeding programs and for elucidating the underlying mechanisms of sex determination in aquaculture species (Liu et al., 2024; Zhang et al., 2021). In the present study, Sanger sequencing was employed to validate the genotypes of control males, neomales, and female broodstock in snakeskin gourami, with particular emphasis on assessing the practical reliability of SNP_19, SNP_20, and SNP_21 as sex-linked molecular markers (Chapter III, VI). The clear and non-overlapping genotype patterns observed among the broodstock groups provide strong support for the applicability of these SNPs in broodstock management and selective breeding programs. Specifically, control males consistently exhibited heterozygosity at SNP_19 (G/T), SNP_20 (C/T), and SNP_21 (G/A), whereas females showed homozygosity at SNP_19 (G/G), SNP_20 (C/C), and SNP_21 (G/G). In contrast, supermales displayed homozygous genotypes at all three loci, namely SNP_19 (T/T), SNP_20 (T/T), and SNP_21 (A/A). This distinct genotype configuration conforms to expectations under an XX/XY sex determination system and complements the inheritance-based evidence presented in Chapters III and VI. Importantly, the present analysis focuses on marker validation at the broodstock level, thereby avoiding redundancy with earlier segregation analyses. Comparable patterns have been widely reported in teleost fish, in which hormonally sex-reversed individuals retain their original chromosomal sex and can be reliably distinguished using sex-linked molecular markers (Devlin & Nagahama, 2002; Piferrer, 2001). The consistency of genotypes observed in neomales across all examined SNP loci indicates that these markers are not affected by hormonal treatment, underscoring their robustness and long-term reliability for breeding applications. In conclusion, the present findings demonstrate that SNP_19, SNP_20, and SNP_21 constitute a reliable and application-ready marker set for genetic sex identification in snakeskin gourami. Moreover, these results provide additional molecular evidence supporting an XX/XY sex determination system in this species. The integration of these SNP markers into selective breeding strategies is expected to enhance the efficiency, predictability, and scalability of all-female population production in snakeskin gourami aquaculture.

5.5.4 Functional confirmation of the XX/XY sex determination system through progeny testing

Building upon the molecular evidence obtained from SNP-based genotyping of broodstock, progeny testing provides an essential phenotypic and functional validation of sex determination in snakeskin gourami. While sex-linked SNP markers reliably distinguish genetic sex at the broodstock level, the evaluation of gonadal development and sex ratios in offspring offers direct confirmation of how these genotypes are translated into reproductive outcomes. In the present study, gonadal sex of progeny derived from control males and supermales was successfully identified using the squash method, with histological analysis serving as a confirmatory approach. The high degree of consistency between squash preparations and histological sections demonstrates that gonadal differentiation in snakeskin gourami is sufficiently advanced at three months post-hatching to allow reliable morphological sex identification. Female progeny displayed characteristic ovarian features, including rounded oocytes in squash preparations and ovaries dominated by oogonia and previtellogenic oocytes in histological sections. Conversely, male progeny exhibited dense clusters of spermatogenic cells in squash preparations and well-organized lobular testes containing multiple stages of spermatogenesis. Similar agreement between squash and histological observations has been reported in other teleost species, supporting the utility of this combined approach for juvenile sex assessment (Guerrero and Shelton, 1974; Wang et al., 2008).

Importantly, progeny testing results further substantiated the genetic sex determination pattern inferred from SNP genotyping. Crosses between control XY males and XX females produced progeny with an approximately balanced sex ratio, consistent with Mendelian expectations under an XX/XY system. In contrast, progeny derived from supermales exhibited a uniform male phenotype, with no females detected. This extreme deviation from the expected 1:1 ratio provides compelling functional evidence that supermales act as fertile phenotypic males and transmit a Y-linked sex-determining factor to all offspring.

From an applied perspective, the successful use of the squash method combined with histological validation offers a practical and efficient strategy for large-scale sex identification in breeding programs. When integrated with the SNP-based

genotyping results presented earlier, these findings establish a clear and coherent link between genotype, phenotype, and reproductive outcome. Such concordance across molecular, histological, and progeny-testing approaches greatly strengthens confidence in the inferred XX/XY sex determination system in snakeskin gourami.

5.6 Conclusion

This study establishes a reliable framework for sex control in snakeskin gourami by integrating hormonal manipulation, molecular genotyping, gonadal histology, and progeny testing. Dietary E2 treatment effectively induced stable phenotypic sex reversal without compromising reproductive capacity. Both neofemales and supermales were fully fertile and produced viable offspring with predictable sex ratios, confirming that hormonal sex reversal alters gonadal phenotype while preserving genetic sex. Sex-specific SNP markers (SNP_19, SNP_20, and SNP_21) consistently distinguished genetic sex among control broodstock, neofemales, and supermales, regardless of phenotypic appearance, demonstrating their robustness for broodstock identification and breeding management. In addition, the combined use of the squash method and histological analysis enabled accurate sex identification in juvenile progeny at three months post-hatching. The strong concordance between these approaches indicates that the squash method is a rapid and practical tool suitable for large-scale progeny sex screening.

Collectively, these results provide compelling molecular, phenotypic, and reproductive evidence supporting an XX/XY sex determination system in snakeskin gourami.

5.7 Acknowledgements:

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CHAPTER VI

OVERALL CONCLUSION AND IMPLICATION

6.1 Overall conclusion

This dissertation systematically investigated the sex determination system and sex control strategies in snakeskin gourami (*Trichopodus pectoralis*) through an integrated framework combining transcriptome-based SNP discovery, multilayer molecular validation, hormonal sex reversal, and functional progeny testing. By linking RNA-level polymorphisms, genomic DNA variants, phenotypic sex differentiation, and inheritance outcomes, this study provides comprehensive molecular, developmental, and reproductive evidence supporting an XX/XY sex determination system in this species.

Firstly, transcriptome sequencing of juvenile and adult gonads enabled large-scale identification of sex-associated nucleotide variants at the RNA level. A total of 21 candidate RNA single-nucleotide polymorphisms (SNPs) were initially screened from ovarian and testicular transcriptomes. These RNA SNPs were further validated by Sanger sequencing and PACE SNP genotyping assays using cDNA templates, confirming their reproducibility and was associated expression patterns across individuals. Among these, nine RNA SNPs located in seven candidate genes (*ift172, man2a1, crk, ezh2, cs, cast,* and *kap3l*) consistently exhibited male-specific heterozygosity and female homozygosity in cDNA samples. Expression profiling revealed distinct sex-biased transcription patterns, with *ift172, man2a1,* and *kap3l* showing male-biased expression, whereas *crk, ezh2,* and *cs* were predominantly expressed in ovaries, suggesting their involvement in gonadal differentiation and maturation.

Subsequent genomic DNA validation revealed that only three of the nine RNA SNPs (SNP_19, SNP_20, and SNP_21) exhibited consistent nucleotide polymorphisms at the genomic level, which were confirmed by both Sanger sequencing and PACE SNP assays and showed stable heterozygosity in males and homozygosity in females, thereby confirming their reliability as true sex-linked DNA markers for genotypic sex

identification. In contrast, the remaining six RNA SNPs were not detected at the genomic level. Together with the observed male-biased expression of the RNA-editing enzyme *adenosine deaminase acting on RNA (ADAR)*, these results strongly suggest that RNA editing contributes to post-transcriptional regulation during sex differentiation in snakeskin gourami. In addition, mismatch-sensitive T7 endonuclease I (T7EI) assays independently confirmed the presence of nucleotide mismatches at SNP_19, SNP_20, and SNP_21, providing enzymatic validation of these sex-associated genomic variants.

Secondly, the validated sex-specific DNA markers were applied to determine the genotypic sex of hormonally manipulated and control (untreated) broodstock. Using marker-assisted genotyping, individuals were accurately classified as genetic females (XX), genetic males (XY), 17α-methyltestosterone (MT)-treated males (XY), and MT-derived neomales (XX). Although MT-treated broodstock exhibited reduced reproductive performance compared with untreated males, neomales retained functional male fertility and produced viable sperm. Controlled breeding experiments demonstrated that crosses between XX neomales and XX females consistently produced 100% female offspring, whereas crosses involving XY males yielded progeny with approximately 1:1 sex ratio. Genotypic analysis of progeny using PACE SNP assays confirmed stable transmission of XX genotypes from neomales, providing functional genetic validation of an XX/XY sex determination system and demonstrating the practical utility of the developed DNA markers for marker-assisted breeding.

Thirdly, functional verification of the sex determination mechanism was further strengthened through the production and characterization of neofemales and supermales. Neofemales induced by dietary 17β-estradiol (E2) treatment exhibited stable ovarian phenotypes while retaining male (XY) genotypes, as confirmed by Sanger sequencing analysis of the sex-specific SNP markers SNP_19, SNP_20, and SNP_21. Progeny testing of neofemales revealed male-biased offspring ratios consistent with Mendelian inheritance under an XX/XY system. Continued rearing and genotypic screening enabled identification of supermales (YY), which were further verified by molecular markers and progeny analysis. Crosses between supermales and wild-type females produced exclusively male offspring, conclusively demonstrating Y-linked transmission of sex-determining factors.

In parallel, phenotypic sex identification using the squash method showed

complete concordance with histological observations at three months post-hatching, indicating that this approach provides a rapid, low-cost, and reliable method for large-scale progeny sex screening. The integration of molecular genotyping, gonadal histology, and progeny testing ensured robust cross-validation among genetic sex, phenotypic sex, and reproductive outcomes.

Collectively, this dissertation establishes a comprehensive and rigorously validated framework for sex identification and sex control in snakeskin gourami. Through sequential verification using transcriptome analysis, Sanger sequencing, PACE SNP genotyping, mismatch-sensitive enzymatic assays, hormonal sex reversal, and controlled breeding experiments, this study provides definitive molecular, phenotypic, and reproductive evidence supporting an XX/XY sex determination system in snakeskin gourami. The sex-specific SNP markers developed herein offer reliable tools for broodstock identification and precision breeding, thereby providing a scientific foundation for sustainable and hormone-reduced all-female production strategies in female-biased aquaculture species.

6.2 Implication

The findings of this dissertation provide important implications for understanding sex determination and differentiation in teleost fishes, particularly in species lacking morphologically differentiated sex chromosomes. By integrating transcriptome-based SNP discovery with genomic validation and progeny testing, this study demonstrates that sex determination in snakeskin gourami follows a male-heterogametic (XX/XY) system. The identification of sex-associated RNA SNPs, together with evidence suggesting RNA editing involvement, expands current perspectives on the regulatory mechanisms underlying fish sex differentiation. These results highlight that sex determination is not governed solely by DNA-level variation but may also involve post-transcriptional regulation, thereby contributing to a more comprehensive understanding of the molecular architecture of sex determination in teleosts.

From a methodological perspective, this study establishes a robust and transferable framework for sex-control research in non-model aquaculture species. The combined use of transcriptomic SNP screening, molecular genotyping, mismatch-sensitive assays, histological verification, and progeny testing provides multi-level

validation of sex-specific markers. Importantly, the successful application of these markers across different developmental stages demonstrates their reliability and practical value. This integrative strategy reduces uncertainty associated with single-method approaches and offers a standardized workflow that can be adapted to other species with complex or unresolved sex determination systems.

In aquaculture applications, the validated sex-specific DNA markers enable accurate identification of neomales, neofemales, and supermales, providing a foundation for hormone-free monosex production. The generation of all-female or all-male populations using genetically verified broodstock addresses concerns related to food safety, environmental sustainability, and regulatory acceptance. Given the female-biased growth advantage of snakeskin gourami, these breeding strategies have strong potential to improve production efficiency and economic value, supporting sustainable and genetically informed aquaculture management.

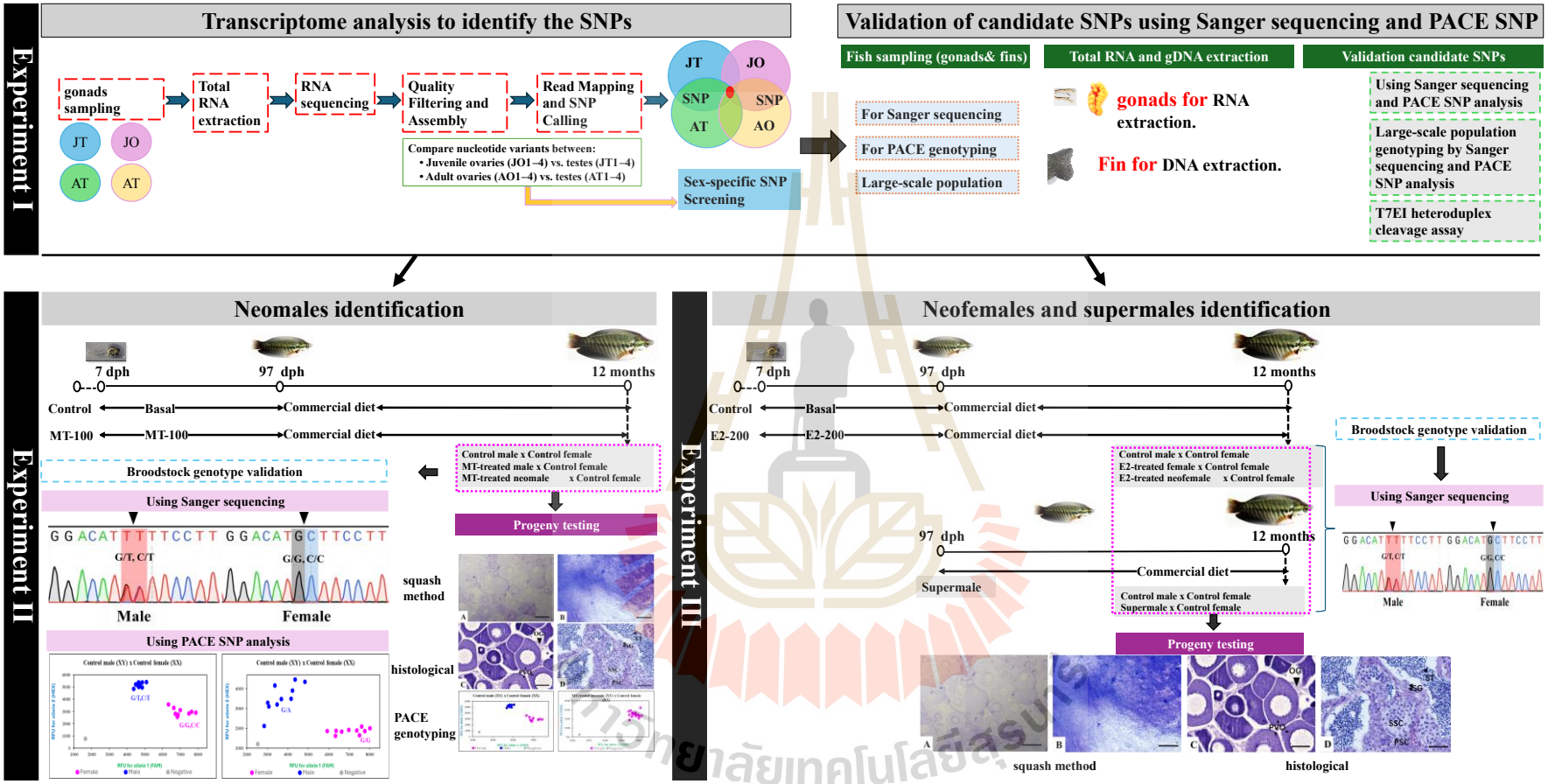

Figure 6.1 Conclusion map.

APPENDIX

The supplementary information (Table S1-S3) related to the transcriptome analysis results is available at the following Google Drive link: https://drive.google.com/drive/folders/1sLSpv5OlKd-QKPe9TRuZGDdf9mOSQXaA?usp=drive_link

SNP_2: Female (A/A); male(A/G)

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                                ctaacattcttccatgaacgagcttcctacagactccgaaaa      4

ATGCAACTGAAGCATCTTAAGACACTGTTAACCCCCCAGGAGGGAGCTGCCAAGGTGACA     102
  M  Q  L  K  H  L  K  T  L  L  T  P  Q  E  G  A  A  K  V  T      20
TGCATGGCCTGGGCCTCCAACAACACCAAGTTCGCTGTTTGCACTGTTGACAGAGTGGTG     162
  C  M  A  W  A  S  N  N  T  K  F  A  V  C  T  V  D  R  V  V      40
CTGCTCTACGATGAGCATGGAGAGAGGAGAGACAAATTCTCCACCAAACCCCTGGACTCC     222
  L  L  Y  D  E  H  G  E  R  R  D  K  F  S  T  K  P  L  D  S      60
AAGTATGGAAAGCAGAGCTACGTTGTGAATGACATGGTGTTCTCACCTGACTCCACCAAA     282
  K  Y  G  K  Q  S  Y  V  V  N  D  M  V  F  S  P  D  S  T  K      80
ATTGCAATCGGGCAGTCCGACAACATCATCTTTGTCTACAGAATTGGAGAGGATTGGGGC     342
  I  A  I  G  Q  S  D  N  I  I  F  V  Y  R  I  G  E  D  W  G     100
GACAAAAAAGCCATTTGCAACAAATTTGTTCAAACAAGTTCTGTGACCTGTCTGCTTTGG     402
  D  K  K  A  I  C  N  K  F  V  Q  T  S  S  V  T  C  L  L  W     120
CCTGCAGAGCATGCCATTGTTTATGGATTAATTGAAGGCAAGGTTCGTCTTGCTAACACT     462
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CATACCAACAAGTCCTCCACCATCTACGGCACAGAGTCCTGTGTCGTTTCACTGGCATCA     522
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  R  E  F  T  V  A  A  T  S  P  S  G  Q  S  V  V  F  G  S  Y     260
GACCGGTTGCGGGTGTTTAACTGGGCTCCGAGAAGAGGTGTGTGGGATGAAGCCAAGCCT     882
  D  R  L  R  V  F  N  W  A  P  R  R  G  V  W  D  E  A  K  P     280
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  F  F  F  E  N  E  T  V  C  M  I  F  N  A  G  E  L  S  L  V     420
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ATCAGTGTGCGACTAAATGAGAGGAAGCAGAGGGGAGCTGAGGACAACAAGAAGCTTGCT    1422
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  Q  N  R  G  S  L  C  I  W  Y  S  I  D  S  P  E  S  I  T  M    560
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ATCCCTGACATCCATCTCAAATACGCCATATACCTGGAAGATGAGGGAAAGTTCTCTGAG   3522
  I  P  D  I  H  L  K  Y  A  I  Y  L  E  D  E  G  K  F  S  E   1160
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  N  K  D  W  A  N  A  Q  R  V  A  E  G  F  D  P  E  S  V  S    1200
GAGGTTCTGGTTAACCAAGCCAAGTTCTGCTTTGAACAGAAAGATTTCCAGAAGGCTGAA    3702
  E  V  L  V  N  Q  A  K  F  C  F  E  Q  K  D  F  Q  K  A  E    1220
GCCTTCCTGCTCCGAGCCCAGAGGCCTGAACTGGCTGTTAAATATTACAAGGATGTAGAC    3762
  A  F  L  L  R  A  Q  R  P  E  L  A  V  K  Y  Y  K  D  V  D    1240
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  M  W  S  D  A  M  R  I  C  K  E  Y  L  P  N  K  L  S  L  L    1260
CAAGAGGAGTACGAGAAGGAGACCTCAAAGAAGGGAAACCGAGGTGTGGAGGGTCTGCTG    3882
  Q  E  E  Y  E  K  E  T  S  K  K  G  N  R  G  V  E  G  L  L    1280
GAACAGGCCCGGGAGTGGGAACAGTCCGGTGAGTATTCCCGGGCCGTGGAGTGTTACCTG    3942
  E  Q  A  R  E  W  E  Q  S  G  E  Y  S  R  A  V  E  C  Y  L    1300
AAGGTGAAGGAGGACTCCAACACGGCTCTGATGGAGAAGTGCTGGATGAAGGCTGCGGAG    4002
  K  V  K  E  D  S  N  T  A  L  M  E  K  C  W  M  K  A  A  E    1320
TTGTCCATCAAGTTCCTCAGTGGAGATCGAGCTGTGGAGGTGGTCCAAGTGGTCGGACCA    4062
  L  S  I  K  F  L  S  G  D  R  A  V  E  V  V  Q  V  V  G  P    1340
CGCCTCACACACCTGAGGAAGTACAACGCTGCGGCTGAGCTCTACTTAAACATGGACCTG    4122
  R  L  T  H  L  R  K  Y  N  A  A  A  E  L  Y  L  N  M  D  L    1360
ATCAAAGAGGCTATCGATGTGTTTATTGAGGGTGAAGAGTGGAACAAAGCCAAGAGAGTT    4182
  I  K  E  A  I  D  V  F  I  E  G  E  E  W  N  K  A  K  R  V    1380
GCTAAGGAGCTGGAGCCAAGGTATGAGGCCTACGTAGACCACAAGTATAAAGAGCATCTG    4242
  A  K  E  L  E  P  R  Y  E  A  Y  V  D  H  K  Y  K  E  H  L    1400
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  K  N  Q  G  K  V  D  S  L  V  G  V  D  V  M  A  A  L  D  M    1420
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  S  S  V  G  K  V  A  N  K  E  D  W  N  K  F  L  M  A  T  K    1720
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  P  A  S  G  F  S  F  Y  *                                     1748
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SNP_3: Female (A/A); male(A/G)

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SNP_4: Female (A/A); male(A/G)

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SNP_5: Female (A/A); male(A/G)

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GAGTTGACAGAGCACCAGCTGCCTGGCGCCCTACCACCTGAATGCACACCGAACATTGAT    1262
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TACAAGCGCAAGAACATGGAGATTCTAGAAGACAGTAAACCCTGCGGAATTGACTGCTAC    1442
  Y  K  R  K  N  M  E  I  L  E  D  S  K  P  C  G  I  D  C  Y     340
ATGTACCTGGTGCAGGATGGGATAGTGAGGAGTATCCCGCAGGTGTGGTTTCAGAGCGA    1502
  M  Y  L  V  Q  D  G  I  V  R  E  Y  P  A  G  V  V  S  E  R     360
GCCAAGACACCCTCCAAACGCACTGTGGGCCGTCGCCGTGGACGACTGCCGAACAGCAAC    1562
  A  K  T  P  S  K  R  T  V  G  R  R  R  G  R  L  P  N  S  N     380
AGCCGTCCCAGCACCCCAACTGTTTCCTCGGAAACCAAAGACACAGACAGTGACCGAGAG    1622
  S  R  P  S  T  P  T  V  S  S  E  T  K  D  T  D  S  D  R  E     400
GGGAGCAAAGAAGACGAGAGAGACAATGACGAATACAAGAAAGATGAGAACACCAGCAGC    1682
  G  S  K  E  D  E  R  D  N  D  E  Y  K  K  D  E  N  T  S  S     420
TCAGAAGGTAACTCTCGCTGTCAAACTCCTGTGAAGATGAAGCTGAACAGTGAGCCTGAA    1742
  S  E  G  N  S  R  C  Q  T  P  V  K  M  K  L  N  S  E  P  E     440
GCCGTGGAATGGAGTGGTGCTGAAGCCTCTTTGTTTAGGGTTCTTATCGGGACATACTAT    1802
  A  V  E  W  S  G  A  E  A  S  L  F  R  V  L  I  G  T  Y  Y     460
GATAACTTCTGTGCCATTGCACGACTCATAGGCACCAAGACCTGCAGACAGGTTTACGAG    1862
  D  N  F  C  A  I  A  R  L  I  G  T  K  T  C  R  Q  V  Y  E     480
TTCAGGGTCAAGGAGTCGAGCATCATCGCCCGGGCTCCAGCTGAAGATGAGGACACACCA    1922
  F  R  V  K  E  S  S  I  I  A  R  A  P  A  E  D  E  D  T  P     500
CCCAGAAAGAAGAAGAGGAAACACAGACTGTGGGCCACTCACTGCAGGAAGATCCAGCTG    1982
  P  R  K  K  K  R  K  H  R  L  W  A  T  H  C  R  K  I  Q  L     520
AAGAAAGATGGCTCATCCAACCATGTATATAATTACCAGCCATGTGACCACCCGCGGCAG    2042
  K  K  D  G  S  S  N  H  V  Y  N  Y  Q  P  C  D  H  P  R  Q     540
CCCTGTGACTCCTCTTGTCCATGCGTCACTGCTCAGAACTTTTGTGAGAAGTTCTGTCAG    2102
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  P  C  D  S  S  C  P  C  V  T  A  Q  N  F  C  E  K  F  C  Q     560
TGCAGCTCTGAGTGTCAGAACCGTTTCCCAGGTTGCCGTTGCAAAGCCCAGTGTAACACT    2162
  C  S  S  E  C  Q  N  R  F  P  G  C  R  C  K  A  Q  C  N  T     580
A/GAGCAGTGCCCCTGCTACCTGGCAGTGAGGGAGTGTGACCCCGACCTTTGCCTGACCTGT    2222
K/E  Q  C  P  C  Y  L  A  V  R  E  C  D  P  D  L  C  L  T  C     600
GGCGCTGCAGACCACTGGGACAGTAAGAACGTGTCCTGCAAGAACTGCTCCATCCAGAGA    2282
  G  A  A  D  H  W  D  S  K  N  V  S  C  K  N  C  S  I  Q  R     620
GGAGCCAAGAAGCACCTGCTGCTGGCCCCTTCAGACGTGGCTGGTTGGGGCATCTTCATC    2342
  G  A  K  K  H  L  L  L  A  P  S  D  V  A  G  W  G  I  F  I     640
AAAGAGCCAGTCCAGAAAAATGAGTTCATCTCAGAGTACTGTGGAGAGATCATCTCTCAG    2402
  K  E  P  V  Q  K  N  E  F  I  S  E  Y  C  G  E  I  I  S  Q     660
GATGAAGCAGACCGCAGAGGCAAAGTCTATGACAAATACATGTGCAGCTTTCTGTTCAAT    2462
  D  E  A  D  R  R  G  K  V  Y  D  K  Y  M  C  S  F  L  F  N     680
CTTAACAATGATTTTGTTGTCGATGCCACAAGAAAAGGCAATAAGATCCGCTTTGCCAAC    2522
  L  N  N  D  F  V  V  D  A  T  R  K  G  N  K  I  R  F  A  N     700
CATTCTGTCAACCCTAACTGCTATGCAAAAGTGATGATGGTGAATGGGGATCACAGGATA    2582
  H  S  V  N  P  N  C  Y  A  K  V  M  M  V  N  G  D  H  R  I     720
GGAATCTTTGCCAAGAGAGCCATCCAGACAGGAGAGGAACTCTTCTTCGACTACAGATAC    2642
  G  I  F  A  K  R  A  I  Q  T  G  E  E  L  F  F  D  Y  R  Y     740
AGCCAGGCTGACGCTCTGAAGTATGTTGGGATTGAGCGGGAGATGGAGATCCCTTGAttg    2702
  S  Q  A  D  A  L  K  Y  V  G  I  E  R  E  M  E  I  P  *        758
tgacttctacctccggtggatcaacaacacaaaggccttacaaacacaaacaaacaccttt    2762
tttttggtatctatcaaaaagctatgatgcttcaggaaaactgggaatagttgcggaaaa    2822
gtttgtttttgtttttttggttgctgtctttagacacttttttgtgtgattctattttat    2882
ttttttcttctttttctttgtcaaatcttccccttctgttggcgggaggttaggatcctt    2942
aaacgtgtcctcagatgaaggtgcagatcaactacaaactgttgtttttggggttttttt    3002
tacgattcctcttcggcgtagtatttcagacaaaccaaggactttttttttgttgttgtt    3062
gttgttcccgtcgctgtttggtttctccggcaaagagaattgaaagtttttcgttgggct    3122
caaggcctgggtagaaacccgagaccagagcaggaggtaaagcaacatccgagcgtacac    3182
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gctcgccacagacagaccacagacgggatacctgaacacttagagaaccaatagggctgt    3302
cgctttgtgtgtttgctgcttgatttaaccgacttcacctttcttgtagctgattagacg    3362
aatagcctcccctgttcgtttgtccccctccgacgtgggcacatccaaactgctggtggt    3422
ttttagtgaaagccccccccccttctttttttctccatcaaggtatatgccatagtacttg    3482
tatttgaagtgatttcgttttttaacttaacattatcttgaacttttttgacatggctgta    3542
gtacatgttgtgtttatttttagagattgttgcaataattcttgtggatgttttatcaga    3602
gtgtgcgtgagtgcgtgcgtgcgtgtgtgtgtgt                              3636
```

SNP_12: Female (A/A); male(A/G)

```
                                                        gcgggcc       7
cgaatccctcttcaaccttctgctttctggaggggcaccttcaaccagctacgcggtgca       67
ctgacagcggctttgtcatttgtaaagaattagttgaaataacccttttacacagacaca      127
ATGTCCTTCCTGACCGCCAGCAGGCTAGCACCCAAACTGCTAAATTCAAAGAATGCCACT      187
  M  S  F  L  T  A  S  R  L  A  P  K  L  L  N  S  K  N  A  T      20
TACTTGTTTGTGGCTGCCAGAAATGCCAGCGCATCAACAACAAATTTGAAGGATGTTCTT      247
  Y  L  F  V  A  A  R  N  A  S  A  S  T  T  N  L  K  D  V  L      40
GCAGATCTTATCCCTAAAGAACAAACAAGGATTAAGAATTTCAAACAACAGTATGGAAAA      307
  A  D  L  I  P  K  E  Q  T  R  I  K  N  F  K  Q  Q  Y  G  K      60
ACCAACATAGGACAGATTACTGTAGACATGGTCTATGGAGGTATGAGAGGAATGAAAGGT      367
  T  N  I  G  Q  I  T  V  D  M  V  Y  G  G  M  R  G  M  K  G      80
CTGGTGTATGAGACATCAGTATTGGATCCTGAGGAGGGCATCCGGTTCCGAGGTTACAGC      427
  L  V  Y  E  T  S  V  L  D  P  E  E  G  I  R  F  R  G  Y  S     100
ATTCCTGAGTGTCAGAAGCTTCTGCCTAGAGCTCCAGGAGGTGAGGAGCCTCTACCTGAA      487
  I  P  E  C  Q  K  L  L  P  R  A  P  G  G  E  E  P  L  P  E     120
GGACTCTTCTGGCTGCTGGTCACTGGACAGGTTCCCACCGAGGAGCAGGTCAACTGGGTG      547
  G  L  F  W  L  L  V  T  G  Q  V  P  T  E  E  Q  V  N  W  V     140
TCCAAAGAATGGGCAAAGAGAGCAGCACTTCCATCTCACGTTGTCACCATGTTGGATAAT      607
  S  K  E  W  A  K  R  A  A  L  P  S  H  V  V  T  M  L  D  N     160
TTCCCTACAAACCTGCACCCAATGTCTCAGTTTAGTGCTGCAATCACAGCTCTAAACAGT      667
  F  P  T  N  L  H  P  M  S  Q  F  S  A  A  I  T  A  L  N  S     180
GAGAGCAGTTTTGCACGGGCGTACTCTGAGGGTGTCCACAAGTCCAAGTACTGGGAGTTT      727
  E  S  S  F  A  R  A  Y  S  E  G  V  H  K  S  K  Y  W  E  F     200
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ATTTATGAAGACTCCATGGACTTAATTGCTAAGCTGCCCTGCATTGCAGCCAAGATCTAT     787
  I  Y  E  D  S  M  D  L  I  A  K  L  P  C  I  A  A  K  I  Y     220
CGCAACCTGTACCGTGAAGGCAGCAGCATTGGAGCAATCGACTCCAACCTGGACTGGTCA     847
  R  N  L  Y  R  E  G  S  S  I  G  A  I  D  S  N  L  D  W  S     240
CACAACTTCACCAACATGCTTGGCTACAGTGATAACCAGTTCACTGAGCTAATGAGGCTC     907
  H  N  F  T  N  M  L  G  Y  S  D  N  Q  F  T  E  L  M  R  L     260
TACCTCACCATCCACAGTGATCACGAAGGAGGCAACGTCAGTGCCCACACTAGCCATTTG     967
  Y  L  T  I  H  S  D  H  E  G  G  N  V  S  A  H  T  S  H  L     280
GTGGGTAGTGCCCTGTCTGACCCCTATCTGTCCTTCAGCGCTGCTATGAACGGTCTTGCA    1027
  V  G  S  A  L  S  D  P  Y  L  S  F  S  A  A  M  N  G  L  A     300
GGTCCTCTACATGGCCTGGCCAATCAGGAGGTGCTCGTGTGGCTAACTGCCCTGCAGAAG    1087
  G  P  L  H  G  L  A  N  Q  E  V  L  V  W  L  T  A  L  Q  K     320
GAGTTGGGAGGAGAGGTGTCAGATGAGAGGATGAGGGATTACATCTGGAACACACTCAAG    1147
  E  L  G  G  E  V  S  D  E  R  M  R  D  Y  I  W  N  T  L  K     340
TCTGGAAGGGTTGTGCCTGGGTATGGTCACGCTGTCCTAAGAAAGACTGACCCACGTTAC    1207
  S  G  R  V  V  P  G  Y  G  H  A  V  L  R  K  T  D  P  R  Y     360
ACCTGCCAGCGCGAGTTTGCCTTGAAGCACTTGCCCAATGACCCAATGTTCAAGTTGGTT    1267
  T  C  Q  R  E  F  A  L  K  H  L  P  N  D  P  M  F  K  L  V     380
TCCCAGCTGTACAAGATTGTTCCCAATGTTCTCCTGGAGCAGGGTAAGGCCAAGAACCCC    1327
  S  Q  L  Y  K  I  V  P  N  V  L  L  E  Q  G  K  A  K  N  P     400
TGGCCCAATGTGGATGCTCACAGTGGAGTTCTGCTGCAGTACTACGGAATGACTGAGATG    1387
  W  P  N  V  D  A  H  S  G  V  L  L  Q  Y  Y  G  M  T  E  M     420
AATTACTACACTGTGCTCTTTGGTGTGTCTCGAGCCCTCGGTGTGCTGGCCCAGCTGGTG    1447
  N  Y  Y  T  V  L  F  G  V  S  R  A  L  G  V  L  A  Q  L  V     440
TGGAGTAGAGCCCTGGGTTTTCCCCTTGAGCGCCCTAAGTCCATGAGCACAGATGGACTA    1507
  W  S  R  A  L  G  F  P  L  E  R  P  K  S  M  S  T  D  G  L     460
ATGTCACTGGTGGGAGTCAAGTCAGGCTGAagaacaaccttaaagatgttgggaggggggg    1567
  M  S  L  V  G  V  K  S  G  *                                   469
gtagtgtgaacgggtggaggagatggtaatatcaagaggcagacgtacgttgacccccctg    1627
tcatttcaaaccccagggattctgtgggattcaaagagacagtaagcttttaacttcatt     1687
tcacaaatgaaggacctttaaattatcacacctagcgttttagttaatcagacaagttt      1747
tcccattgtgtcgccatatttggaactggcaatgtgaatgcatcacacacatgcctccag     1807
cagtaggctcaaactgttctgttctatgtctacatgatgattttcccctgtgcagtgcct     1867
gcgtgtttgtcattgagcactgggtctgatctataaatatagtggcattcaaagggaact     1927
gtgggagtgaactacacttgattgtgtttgtttaaaaatctaaagttaataactcagcc      1987
ttttgctcctgtggctaatagaatgatttgtttgaattattattcagctcatttttatttc    2047
ttctgtccagtgttcctaacctttttccacccagtctgtcaatgcatttcactcccattt     2107
actctatgaacactccactttaaaagtaaaggtccttagcagtaaacttgacaagctaat     2167
caagagaaacaaactataaaaccatagttaaaagatactgccccttta/gaatctttggt     2227
ctccctttttcaaagttgtatttttctttttattcaaataattaaatgacctctatgatc     2287
aaaataatttttgtctatgtagaagtaatttcccttagctccttttttgttaacatgcgtac    2347
ctttttaacgggtctctctccggtggttttctgagacacagagaactaaaaatgaagtac     2407
agcaacagtaatgtacagtagtgataaagacttgagtaaagggtgttgtacttggcaacc     2467
tgtggtaaatgcttaagagcaaggttgaatgaaaagtgggacagatgggggggggggggt     2525
```

SNP_15: Female (A/A); male(A/G)

```
cacacacacacacacaaacatgaacagacacacacgcacacacacatatacgtatatatc      60
tgtgtgcagcgccgtttttacctggttttacatttaatggaggttcagaagtttgtggat     120
tctttctttgaggacaatgaaaagcagacttcatataacaatacggatgtagagcatgag     180
tgatgaagacagacagaaaggcggtaacctgcgcttaaaaaggggtttaagaaaggtttc     240
gtcgtgccgagggaaggaaggcgctgctcacctccttgcatgtacatatgagctgacagt
300
ctctgtttttccaataaaacactaatgtcgcaacccagagatcatctcagctggcagttt     360
a/ggtgaggtttgttggaaagatggccactgtgtgtgtgagcgtgtgtgtttgtttgttt     420
gtgtgtttaggatgaatgcagagtgtgtgtacagtatcgggatgtttgtgcaatcttgat     480
gtacacagaaaagttatctttaagaacgtggtctgatgacactacaacactagtgtacag     540
cttagtttgcttttatttggacagcacgtggtttacattgttaaatatgtggaggattt      600
cttttatttctgctttgttctgtagaggagagcttctccaaaatgcctttcactttctgt     660
ggagaaatatctttctagagaagggtctttttttttttttttatgtgtttggccttaccga    720
tgtccacaagtgcacttagtgaaccgaggtggtttcactttgtaagccacccatttataa     780
aataaatatactgtaaaaaaataaa
```

SNP_19, 20: Female (GC/GC); male (GC/TT)

SNP_21: Female (G/G); male(G/A)

```
                                                    ccaaaggagaacgtcgccg     19
      ctcctccatagtgctgcttgtgtgtgtgcatcggtgactagcacacagatttgaatgttc      79
      gtgatttaactgtaaaccgtttataaaatcctcttcacatcgtcgttgaatttgaggaag     139
      ATGGAGGATGATGCGCGCTACCTGAAACGCAAAGTGACTGCGGGCAGTTTGGATGTCCAC     199
        M  E  D  D  A  R  Y  L  K  R  K  V  T  A  G  S  L  D  V  H      20
      CCCACGGAGAATGCCCTTGTGGTCCACTATGAGGTGGAAGCGTCCATACTAGGAGAGAGT     259
        P  T  E  N  A  L  V  V  H  Y  E  V  E  A  S  I  L  G  E  S      40
      GGAGGTTGTATGGTGGGGGAACGCAAGGAGGGACAGAAAATTGTCCGTGTCAAAAGTCTT     319
        G  G  C  M  V  G  E  R  K  E  G  Q  K  I  V  R  V  K  S  L      60
      TCTCGTAGCACTGATGTGACAGCTTTGGCCAAGAAGGTGGTGGAGGAGTGTAAGCTCATC     379
        S  R  S  T  D  V  T  A  L  A  K  K  V  V  E  E  C  K  L  I      80
      CCTTCTTCCCGCTTGCCCCAGGTGGAGCAGCTTTTGTACTACTTGCAAAACAGGAAATCT     439
        P  S  S  R  L  P  Q  V  E  Q  L  L  Y  Y  L  Q  N  R  K  S     100
      TCTCCAGTGGTGGGCAAAGTGGATAAGAAGGAGAAGAAAACTCTGAAACCCAGAGAGCTG     499
        S  P  V  V  G  K  V  D  K  K  E  K  K  T  L  K  P  R  E  L     120
      ACACCATTTGAAGGAATTGAGTTAAATGAGGAAGCCTGTATAACCAGAGTGGATGAGTAT     559
        T  P  F  E  G  I  E  L  N  E  E  A  C  I  T  R  V  D  E  Y     140
      GTTGAGCTTCTCTATGAGGACATCCCAGACAAGATCAGAGGCTGCGCTCTAATTCTACAA     619
        V  E  L  L  Y  E  D  I  P  D  K  I  R  G  C  A  L  I  L  Q     160
      CTTGCCCGCAACCCAGACAACCTGGATGAGCTGCTTCAAAATGAGACAGCTCTAGGGGCT     679
        L  A  R  N  P  D  N  L  D  E  L  L  Q  N  E  T  A  L  G  A     180
      CTGGCCAGAGTACTGAGGGAGGATTGGAAACATAGTGTGGAGCTGGCCACCATTATCTTG     739
        L  A  R  V  L  R  E  D  W  K  H  S  V  E  L  A  T  I  I  L     200
    TACATCTTTTTCTGCTTCTCTAGTTTCTCCCAG/ATTTCATGGAGTGGTGAGTCATTATAAA     799
      Y  I  F  F  C  F  S  S  F  S  Q/Q  F  H  G  V  V  S  H  Y  K     220
      ATAGGTGCATTGTGTATGAGTGTTGTGGAACATGAGCTGAAGCGACATGACGTGTGGCGT     859
        I  G  A  L  C  M  S  V  V  E  H  E  L  K  R  H  D  V  W  R     240
      GAGGAGCTACACAAGAAGAACAAAGCTTGTGAGTCAGCACCAGAGAATGGCTCCTTAAGA     919
        E  E  L  H  K  K  N  K  A  C  E  S  A  P  E  N  G  S  L  R     260
      AGAGACCAAGGCAAAGCTCTAAAGAAATACCAAGGCCTTCTGGCCAAACAGGAGCAGCTC     979
        R  D  Q  G  K  A  L  K  K  Y  Q  G  L  L  A  K  Q  E  Q  L     280
      ATTAGAGTGTCTCTTTACCTGTTGTTAAACCTTGCTGAAGACACAAGAACGGAGCTGAAG    1039
        I  R  V  S  L  Y  L  L  L  N  L  A  E  D  T  R  T  E  L  K     600
      ATGAGGAATAAAAACATTGTTGGTCTGTTGGTCAGGGTTCTTGAACGGGACGATGAGGAG    1099
        M  R  N  K  N  I  V  G  L  L  V  R  V  L  E  R  D  D  E  E     320
      CTTCTGGTTCTGGTGGTTACATTCCTCAAGAAACTGTCCATCTTTTTGGAGAATAAAAAT    1159
        L  L  V  L  V  V  T  F  L  K  K  L  S  I  F  L  E  N  K  N     340
      GACATGGCTGAGGTGGATACTATTGAACGCTTGGCTCGCCTTGTTCCCTGTGACCATGAA    1219
        D  M  A  E  V  D  T  I  E  R  L  A  R  L  V  P  C  D  H  E     360
      TATTTGCTTAACCTGATTTTGCGTCTGCTTCTAAACTTGTCTTTTGACTCTGGACTCAGA    1279
        Y  L  L  N  L  I  L  R  L  L  L  N  L  S  F  D  S  G  L  R     380
      GCAAAGATGGTGGAAGCTGGATTGTTACCTAAACTATGTAACTTGTTAGGTGATGAGAAT    1339
        A  K  M  V  E  A  G  L  L  P  K  L  C  N  L  L  G  D  E  N     400
      AACCGTCTAGTAGCCATGCGTATCCTTTATCACATCAGCATCGACGATCGTTTTAAGGGC    1399
        N  R  L  V  A  M  R  I  L  Y  H  I  S  I  D  D  R  F  K  G     420
      ATGTTTGTTTACACTGACTGCATACCCCAGCTTATGCAAATGTTGTTTGAAAATGGTGAG    1459
        M  F  V  Y  T  D  C  I  P  Q  L  M  Q  M  L  F  E  N  G  E     440
      GAGGAAAATGAAGCTGAGCTCATCTCTATCCTCATCAACCTGGCTGTAAACAAGAAGAAT    1519
        E  E  N  E  A  E  L  I  S  I  L  I  N  L  A  V  N  K  K  N     460
      GCACAAGTTATGTGTGAAGGAAATGGATTGAAGATGCTGATGAAAAGGGCTTTGAAGATA    1579
        A  Q  V  M  C  E  G  N  G  L  K  M  L  M  K  R  A  L  K  I     480
      AAAGATAGCCTGCTAATGAAAATGATAAGAAACATTTCACAACATGATGGAGCAACCAAA    1639
        K  D  S  L  L  M  K  M  I  R  N  I  S  Q  H  D  G  A  T  K     500
      CCACTGTTCATAGACTATGTTGGTGACCTGGCAGCAGAGATCCGTGCAGAGGAAGAAGAG    1699
        P  L  F  I  D  Y  V  G  D  L  A  A  E  I  R  A  E  E  E  E     520
      GAGTGGGTTCTGGAGTGTCTGGGGACTTTGGCTAACCTTACCATTCCTGACCTAGATTGG    1759
        E  W  V  L  E  C  L  G  T  L  A  N  L  T  I  P  D  L  D  W     540
      GAGTTAGTGCTCAAGGAGTACAACCTAGTACCTTACCTGAAAGACAGACTTGTACCAGGT    1819
        E  L  V  L  K  E  Y  N  L  V  P  Y  L  K  D  R  L  V  P  G     560
      TCAGCAGAGGATGACCTCATCCTTGAGGTTGTAATTTTGATTGGAACTGTTTCCATGGAT    1879
        S  A  E  D  D  L  I  L  E  V  V  I  L  I  G  T  V  S  M  D     580
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GATGCCTGTGCTGCCATGTTGGCTAAATCTGGCATTATCCCGGCACTTATTGAGTTACTA    1939
  D  A  C  A  A  M  L  A  K  S  G  I  I  P  A  L  I  E  L  L    600
AATGTCCAGCAAGAGGATGATGAGTTTGTGTGTCAGATTGTCTACGTCTTCTATCAGATG    1999
  N  V  Q  Q  E  D  D  E  F  V  C  Q  I  V  Y  V  F  Y  Q  M    620
GTTTTTCACCAAGCTACACGTGACGTCATAATCAAAGATACCCTTGCTCCAGCCTACCTT    2059
  V  F  H  Q  A  T  R  D  V  I  I  K  D  T  L  A  P  A  Y  L    640
ATAGATCTGATGCACGACAAGAACACTGAAATTAGAAAAGTGTGTGACAACACCCTCGAT    2119
  I  D  L  M  H  D  K  N  T  E  I  R  K  V  C  D  N  T  L  D    660
ATTATTGCTGAATACGATGAACAGTGGGGGAGGAAGATTCAGTCAGAAAAGTTTCGTTTT    2179
  I  I  A  E  Y  D  E  Q  W  G  R  K  I  Q  S  E  K  F  R  F    680
TATAACAACCAGTGGTTGGAGATGGTTGAAAGTCGACAGGCTGATGAGTCTGAGCCATAT    2239
  Y  N  N  Q  W  L  E  M  V  E  S  R  Q  A  D  E  S  E  P  Y    700
CTCTATGATAACGACAATGACAGGACGGATCTGTTCTACAGCGCAGATGGAATAACTCCA    2299
  L  Y  D  N  D  N  D  R  T  D  L  F  Y  S  A  D  G  I  T  P    720
GGAGATGGTTCTATCAGTCCAGACTTCTTCTGTGATCCACAGACACAAAATGGAGATTCA    2359
  G  D  G  S  I  S  P  D  F  F  C  D  P  Q  T  Q  N  G  D  S    740
CATCTTACAGGAGACAATGATGTCTTCGACCAGACAAGCTCATCGCCTGGAAGACCGGCT    2419
  H  L  T  G  D  N  D  V  F  D  Q  T  S  S  S  P  G  R  P  A    760
ACTGCTTATGGCTTTAGACCCGATGAACAGCCCTCCTATCAGTATTCAAAGACCCACACA    2479
  T  A  Y  G  F  R  P  D  E  Q  P  S  Y  Q  Y  S  K  T  H  T    780
CATCTGGCTTGTTAAataatttaggaaattacattattcctctttgtctttgtctttgtc    2539
  H  L  A  C  *                                                  784
attttctcgtcacatcttgcatgtttgggctctagtatcgtttcattgacctttactac     2599
accacattgcattggtcatacagaaccaataagaagctgaaatacaaaatgctaataagt    2659
acagtctttacactagttatttaaaaactgcagttccatgcagtcatttgtttatcatta    2719
tgttaaccaaatattgcatacaggtacagggtcttactttcattcagctcctagtagggt    2779
caacaaatataattataaacaataagtattaacacaggtttagcatgttttcacaggtaa    2839
tattaatatatgttttttttcttatttatttaaaaacattccagtgcttgaatttcgaat    2899
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acctcagtactagagagccacgattgcttatttactgtccctgctctgcccactgctgat    3019
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aaagggagcttaggacatttaatgcattagtagtaacgtgtgtcagtgtatgctcgtaag    3319
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tcatgcatcctggtaagctttatgcttactggataaatactttaaagtgtccaaaatact    4219
actgaaaatgtgtatctgtctttatgtaaatgcgtaagtgtttgcactaaagttataaaa    4279
catgcataatacatcattatggcgttaaaaaaaacttctttgacgccagtcagaatgaa    4339
tccaataaactgaagtagctttgaa                                      4359
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Figure S1 Nucleotide sequences of SNP_2, SNP_3, SNP_4, SNP_5, SNP_12, SNP_15, SNP_19, SNP_20, and SNP_21. Lowercase letters indicate the 5′ and 3′ untranslated regions (UTRs). Underlined letters indicate the stop codon. SNP positions are highlighted in red. Note that SNP_4 and SNP_15 nucleotide sequences contain only 3'UTR.

BIOGRAPHY

Minghua Zhang was born on June 16th, 1992, in Guizhou, China. In June 2018, he received his bachelor's degree in veterinary medicine from the College of Agriculture, Liaodong University. In June 2021, he obtained his Master of Veterinary Medicine from the College of Animal Science, Guizhou University. In March 2022, he began his Doctor of Philosophy (Ph.D.) studies in Animal Production Technology at the School of Animal Technology and Innovation, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima, Thailand, supported by the One Research One Graduate (OROG) scholarship program.