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.

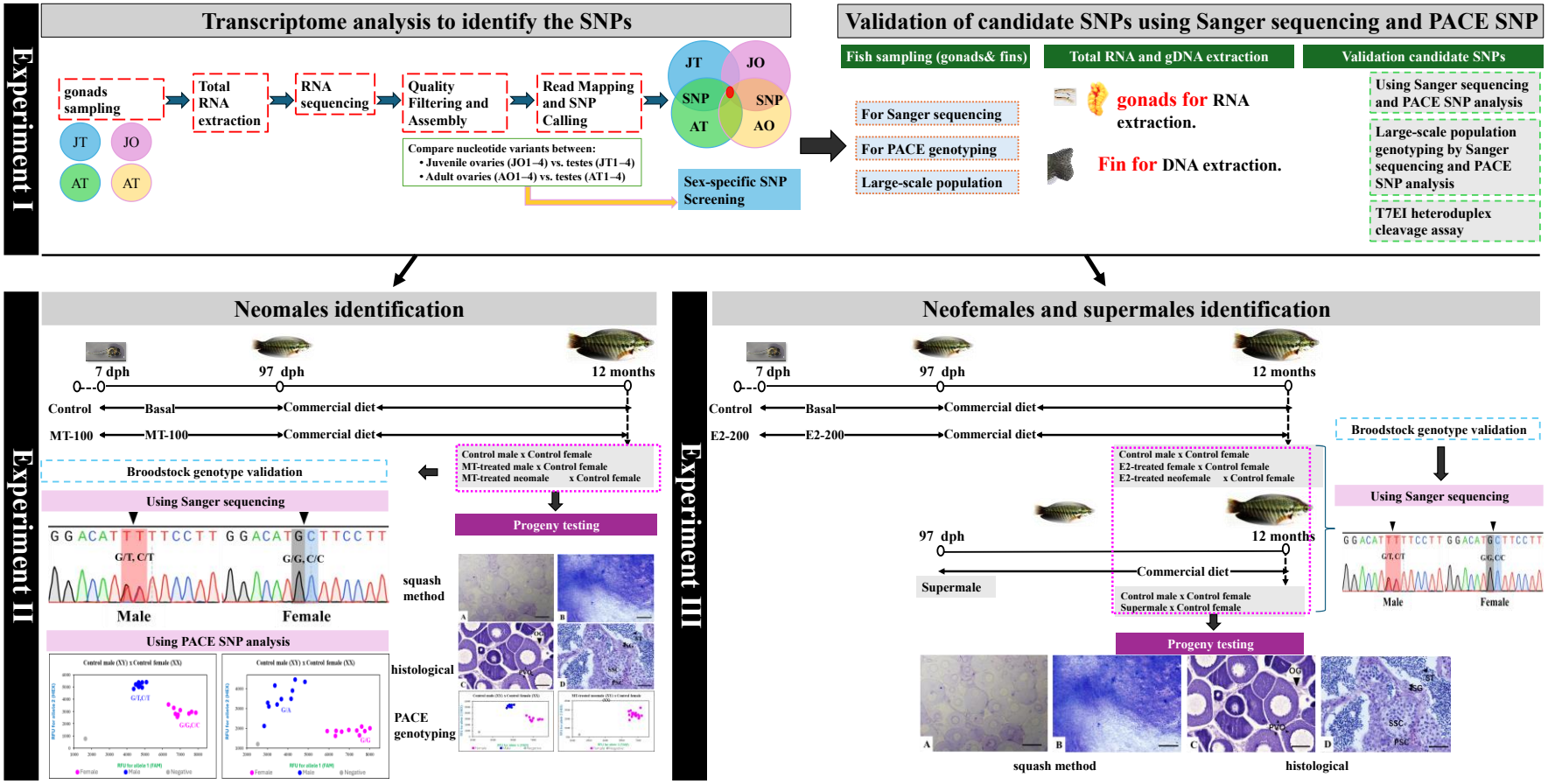

Figure 6.1 Conclusion map.