Single Locus Maintains Large Variation of Sex Reversal in Half-Smooth Tongue Sole (*Cynoglossus semilaevis*)

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**ABSTRACT** Sex determination is a fundamental biological process for individual sex development and population sex ratios. However, for some species, the primary sex might be altered during development, and individuals can develop into the opposite sex. Sex reversal may happen in insects, reptiles, amphibians, and fishes. In half-smooth tongue sole (*Cynoglossus semilaevis*), some genetically female fish irreversibly reverse to pseudomales, resulting in higher costs in aquaculture owing to a lower growth rate of male fish during a 2-yr growth period. Here, we identified a locus with large controlling effect on sex reversal in the half-smooth tongue sole through genome-wide association study with high-density single nucleotide polymorphisms (SNPs). This SNP is located at the third intron of the F-box and leucine rich repeat protein 17 (*FBXL17*) gene on the Z chromosome, and it has two alleles, A and T. Genetic females with ZAW genotypes will never reverse into phenotypic males, but those with ZTW genotypes can sometimes undergo sex reversal. This SNP explains 82.7% of the genetic variation, or 58.4% of the phenotypic variation. Based on our results, a reproductive management program could be developed to improve the phenotypic female ratio in aquaculture, and elucidate the mechanism of sex reversal in half-smooth tongue sole. We expect that these findings will have a substantial impact on the population management in many harvested species where sex reversal occurs.

**KEYWORDS**

sex reversal, genome-wide association study, half-smooth tongue sole, *FBXL17*
In tongue sole aquaculture, most farms can control the temperature relatively well to 22°C, but not all genetic female offspring of the same family, and under the same environment, reversed to pseudomales; therefore, this dichotomy implies the genetic difference between reversed and nonreversed fish, and it was speculated that genes on the Z chromosome may contribute to sex reversal (S. Chen et al. 2014). Therefore, it is necessary to investigate if there are variants controlling sex reversal, and if we can genetically control sex reversal in tongue sole aquaculture. In the present study, we obtained the sex reversal phenotype using tissue sections combined with molecular markers, and performed a genome-wide association study (GWAS) to detect the genetic locus associated with sex reversal using an efficient generalized linear mixed model (Kang et al. 2010).

### MATERIALS AND METHODS

#### Materials

In March 2013, six adult females and 11 phenotypic males of half-smooth tongue sole were randomly selected and tagged as the parents, which were cultivated in Dongying, Shandong Province, China. These parental fish were raised in the same pond for reproduction. The larvae were transferred to another pond 36 hr after hatching. Since sex reversal of half-smooth tongue sole usually happens in the first 90 d in early stage, so this experiment was designed to last for 90 d at a constant temperature of 22°C to avoid additional environmental effects. Thereafter, 268 fry were randomly selected for DNA extraction, parentage analysis, and genetic and phenotypic sex detection. Finally, 115 genetic females were used for GWAS. In April 2014, 399 fish raised under the same conditions as those in 2013, with no parental information, were randomly harvested from the same farm for confirmation of our detected polymorphism associated with sex reversal.

#### Phenotypes

Fins and gonads were sampled, and fins were used for DNA extraction. The genetic sex was determined using the method of Chen et al. (2009), and all

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### Table 1 Primers for parentage analysis

| Primer Name | Primer Sequence (5'–3') | Code |
|-------------|-------------------------|------|
| 1-F         | CCCAGACGAGCTTAATC          | JN902297 |
| 2-F         | GCCGTAACAGGTGTTCAACA       |      |
| 3-F         | CGACGGCGTATGTTGAC          | JN902320 |
| 4-F         | CAAGACCCGATGTTGCTTCT        |      |
| 5-F         | GACTCTTCATCGACTGGAAGAGAG   | JN902384 |
| 6-F         | AGTCTGGCCTGACAGGTTT        | JN902469 |
| 7-F         | AGCTGCCATGCTGGAGGAGG       |      |
| 8-F         | CCAGAAAACACTGGACACTCAC     | JN902541 |
| 9-F         | CTGGTGTTCTCGTGGATTGCTT     | JN902587 |
| 10-F        | AGGAGAACAGGTCATGGATGC      | JN902640 |
| 11-F        | AGCTACCAAGGTGCTGACCAC      | JN902723 |
| 12-F        | TCGACGGCGTATGTTGCTTCT       |      |
| 13-F        | AGGAGAACAGGTCATGGATGC      | JN902723 |
| 14-F        | AGCTACCAAGGTGCTGACCAC      | JN902723 |
| 15-F        | TTATATATGCGGAGAAGCTC       | HM060584 |
| 16-F        | GACGACGACGTTGATTGCTT       | EU907031 |
| 17-F        | CTCGACCAAGGTGCTGACCAC      | JN902723 |
| 18-F        | AGGACTTACATGCAGGTCAAGC     | JN902723 |
| 19-F        | CGGACTGTATGCTGGACTT        | JN902723 |
| 20-F        | CATGAGAAGGTTGCTGACG        | JN902723 |
| 21-F        | AGCCGAGATCTGACCATCAGG      | JN902795 |
| 22-F        | GCCTGCATACAAAGGACAGC       |      |

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### Table 2 The SNP information

| Chromosome | Number of SNPs | Span of SNPs (Mb) |
|------------|----------------|------------------|
| 1          | 1573           | 34.47            |
| 2          | 846            | 20.05            |
| 3          | 747            | 16.24            |
| 4          | 876            | 19.90            |
| 5          | 977            | 19.17            |
| 6          | 882            | 18.82            |
| 7          | 734            | 13.76            |
| 8          | 1276           | 30.11            |
| 9          | 829            | 19.60            |
| 10         | 905            | 20.92            |
| 11         | 841            | 20.43            |
| 12         | 748            | 18.30            |
| 13         | 806            | 21.79            |
| 14         | 1081           | 28.83            |
| 15         | 734            | 19.88            |
| 16         | 792            | 18.65            |
| 17         | 686            | 16.46            |
| 18         | 532            | 14.94            |
| 19         | 677            | 17.72            |
| 20         | 705            | 15.15            |
| W          | 110            | 16.17            |
| Z          | 261            | 21.40            |

n: The T<sub>n</sub> for all pair of primers is 56°.
the samples were assigned parents with 21 pairs of SSR primers (Song et al. 2012) (Table 1) using Cervus 3.0 software (Slater et al. 2000) with a Maximum Likelihood approach; only individuals with 99.99% liability were reported. Thereafter, gonads of genetic females were used for phenotypic sex determination by tissue section (Supplemental Material, File S1).

Sex determination by tissue section (Supplemental Material, File S1).

Thereafter, gonads of genetic females were used for phenotypic sex determination by tissue section (Supplemental Material, File S1). The samples were stained in A solution for 5–10 min, then in water for 10 min until the water became blue, then for another 3 min in fresh water; next they were washed in hydrochloric acid solution (1 ml concentrated hydrochloric acid dissolved in 99 ml 70% ethyl alcohol) for several seconds until the samples became light red. Samples were washed in water for 3 min until the water became blue. The samples were then placed in gradient ethyl alcohol for 2 min respectively for each step, from 30, 50, and 70, and finally 80%. The samples were then stained in eosin solution for ~20 sec, and placed in the gradient ethyl alcohol for 2 min per step, from 90, 95, 100, and 100% ethyl alcohol, then benzene alcohol for 2 min, and xylene for 2 min twice.

The samples were observed and analyzed using an Olympus microscope.

Genotypes

One hundred and fifteen genetic females were genotyped using the 2b-RAD method from the Oebiotech Co. Ltd. (Shanghai, China). The 2b-RAD libraries were prepared with BsaXI following the reported protocol (Wang et al. 2012), and were subjected to single-end sequencing using an Illumina HiSeq2500 platform. Using sequencing, a total of 612,818,007 reads was produced, averaging 5,328,852 reads per sample. The 2b-RAD genotyping were performed with the RADtyping program v1.5 (Fu et al. 2013). Reads with no restriction sites, or containing ambiguous base calls (N), long homopolymer regions (>10 bp), excessive numbers of low quality positions (>10 positions with quality of <20) were removed. Finally, 138,666 unique tags were obtained, an average level of coverage was 16× per sample. Totally 66,563 SNP genotypes were obtained and mapped to C. semilaevis genome (S. Chen et al. 2013). The SNPs with minor allele frequencies of <5% or a call rate of <0.90 were discarded, and the remaining missing data were imputed through linkage disequilibrium with 10 closest neighboring markers (see File S2 for the source code). After the quality control, the final genotypic data (File S3 and File S4) in the analysis consisted of 17,618 SNPs without missing genotypes (Table 2).

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**Table 3 The different sex statistics based on parentage analysis**

| Family | Sire* | Dam | Female | Pseudomale | Male | Total |
|--------|-------|-----|--------|------------|------|-------|
| 1      | S1    | D10 | 22     | 1          | 22   | 45    |
| 2      | S12   | D10 | 3      | 4          | 6    | 13    |
| 3      | S14   | D10 | 0      | 10         | 9    | 19    |
| 4      | S16   | D15 | 1      | 1          | 4    | 6     |
| 5      | S9    | D15 | 0      | 14         | 13   | 27    |
| 6      | S19   | D17 | 4      | 3          | 8    | 15    |
| 7      | S38   | D3  | 4      | 0          | 6    | 10    |
| 8      | S39   | D3  | 2      | 3          | 7    | 12    |
| 9      | S40   | D3  | 1      | 3          | 5    | 9     |
| 10     | S2    | D7  | 6      | 12         | 16   | 34    |
| 11     | S11   | D13 | 23     | 15         | 40   | 78    |
| SUM    |       |     | 66     | 66         | 136  | 268   |

*Sire S9 and S14 were pseudomales.

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**Figure 1** Sex differentiation of the half-smooth tongue sole for pseudomales. (A) Ovary and oocytes (×100). (B) Chimeric gonad during the late process of sex reversal from ZW female to pseudomale, mainly composed of spermatangia; the red triangle indicates the oocytes (×100). (C) Testis and sperm cells (×100). (D) Chimeric gonad during the early stage of sex reversal from ZW females to pseudomale; the green triangle indicates the sperm cell (×100).
Statistical analysis
Sex reversal was considered a binomial trait, and the incidence of sex reversal was recorded as one, and the others as zero. We used a logistic animal model to perform an association analysis:

\[
\text{logit}(y) = \mu + b + g + e \quad (1)
\]

where \[\text{logit}(y) = \frac{p}{1-p}\]; \(p\) is the frequency of sex reversal; \(m\) is SNP genotype, and was taken as a fixed effect; \(b\) is the allele substitution effect; \(g\) is the additive effect and follows the distribution \(N(0, \sigma^2_g)\); \(K\) is the realized genetic relationship matrix; and \(e\) is the random error and follows the distribution of \(N(0, \sigma^2_e)\).

Construction
\(K\) follows these procedures, (1) code the genotype matrix \(M\) for each marker, 0 for homozygotes, 1 for heterozygote, and 2 for the other homozygote; (2) standardize \(M\) for each SNP as \(Z\), (3) calculate \(K\) as \(ZZ' / n_m\), where \(n_m\) is the number of markers. An association analysis of each SNP was conducted by comparing the full to the null model:

\[
\text{logit}(y) = \mu + g + e \quad (2)
\]

and the \(-2\)log-likelihood test statistics approximately followed the Chi-square distribution with a freedom of degree of 1. To improve the computation speed, a method similar to that of the efficient multi-locus mixed model (Segura et al. 2012) was used. First, DMU (Madsen and Jensen 2002) was employed to estimate the variance components, \(\sigma^2_g\) and \(\sigma^2_e\); then the linear mixed model was transformed to a simple linear regression through the Cholesky decomposition of the phenotypic (co)variance structure \(V\) as the following equation:

\[
y^* = bm^* + e^* \quad (3)
\]

where \(y^* = V^{-1/2} \cdot \text{logit}(y)\), \(M^* = V^{-1/2} \cdot M\), and \(V = K' \sigma^2_g + \Gamma' \sigma^2_e\); \(m^*\) is the corresponding SNP column of the \(M^*\) matrix. After a genome scan, the threshold value was determined (Piepho 2001).

Table 4 Primers to amplify target fragments

| Sequence (5'-3') | Length | Tm (°C) | GC (%) |
|------------------|--------|---------|--------|
| Forward primer   | CAGATAGCCAGCCTTAGCCC | 21 | 61 | 57.14 |
| Reverse primer   | CCTGTTGTGAGTGGAGTGG | 21 | 61 | 57.14 |
| Product length   | 746    |         |        |
and, once a genetic locus was detected, the corresponding confidence interval was calculated with Li’s method (Li 2011).

**Data availability**

File S1 is phenotypic file containing id, sire, dam, genetic sex, phenotypic sex, and incidence of sex reversal. File S2 contains the source code for miss genotype imputation. File S3 contains genotypes (coded as 0, 1 and 2) of 115 samples, and File S4 is the SNP information.

**RESULTS**

**Sex determination and parentage analysis**

Using sex-specific markers (Chen et al. 2009), 132 genetic females and 136 males were determined, and, among the genetic males, 66 were determined as pseudomale (Table 3). Normal females (Figure 1A), normal males (Figure 1C), and pseudomales (Figure 1, B and D) in reversal were easily distinguished using tissue sections. Before sex reversal was completed, the ZW fish showed a chimeric gonad; after sex reversal was completed, the gonad developed as that of normal males. Parentage analysis showed that these fish came from 11 families, among which the sires of two families were pseudomales. All the ZW offspring of these two pseudomale families reversed into phenotypic males without any exception.

**Genome-wide association study**

GWAS was conducted with all the ZW individuals (except for 17 individuals that failed to genotype with 2b-RAD), and the single SNP at 6,676,874 bp on the Z chromosome (named as Cyn_Z_6676874) was found to be strongly associated with sex reversal in half-smooth tongue sole (\( P < 1.0 \times 10^{-7} \); Figure 2A). The SNP Cyn_Z_6676874 was located at the third intron of the F-box and leucine rich repeat protein 17 (FBXL17) gene, which is the substrate-recognition component of the SCF (SKP1-CUL1-F-box protein)-type E3 ubiquitin ligase complex, interacting with ubiquitination targets through other protein interaction domains (Jin et al. 2004), and the 95% confidence interval was from 6,337,129 to 7,126,693 bp (Figure 2B), where eight known genes were harbored (Figure 2C). Cyn_Z_6676874 explained 82.7% of the genetic variation (the variance components are \( \sigma^2_g = 1.36 \), \( \sigma^2_{sep} = 6.54 \), and \( \sigma^2_e = (\pi^2/3) = 3.29 \), respectively), or 58.4% of the phenotypic variation; it had two alleles, A and T, and when the genotype was ZAW, none of genetic females reversed, whereas for the ZTW genotype, some of the genetic females reversed.

**Confirmation of genetic locus**

To confirm SNP Cyn_Z_6676874, we designed primers (Table 4) to amplify the target fragment containing this SNP, and tested it by sequencing in the population sampled in 2014. Among 399 samples, 196 individuals were genetic females, including 95 ZAW and 101 ZTW genotypes. Tissue sections showed that none of the ZAW individuals reversed, whereas for the ZTW genotype, 52 individuals reversed into phenotypic males.

**DISCUSSION**

Sex reversal has usually been considered an adaptive strategy for animals in evolution (Shapiro 1987; Ghiselin 1974). According Darwin’s theory of evolution, sex reversal must have a genetic basis. The findings in
P. viticeps showed that genetic background was related to sex reversal to some extent (Holleley et al. 2015), whereas, in the half-smooth tongue sole, it was originally thought that temperature was the critical factor affecting the sex ratio. However, in the present study, the locus Cys_Z_6676874 indicated that the genetic background was the primary basis of sex reversal. This is not in conflict with other reports (S. Chen et al. 2014), which showed that higher temperature lead to lower female ratio, because first, our study removed the environmental effects in the experimental design, and it was unnecessary and impossible to repeat earlier experiments; second, we speculated that only Z^W individuals were sensitive to temperature, and the female ratio was sensitive to temperature at the population level, especially if the parental genotypes were missing. Our findings suggest that a more detailed experimental design based on the sex reversal locus is important, and necessary, for the further study of temperature-dependent sex determination. Methylation was also thought to cause sex reversal, because the methylation level of pseudomales was higher than that of normal females, and similar to that of normal males (Shao et al. 2014). Two pseudomale families also showed possible imprinting occurring for pseudomales, which was inherited by the offspring, because all of them reversed into phenotypic males. Regardless of whether methylation is a cause or a result of sex reversal, the sex reversal locus we detected illuminated the mechanism of sex determination of half-smooth tongue sole.

Besides FBXL17, there are seven known genes located within the confidence interval of the sex reversal locus: AhpC/TSA Antioxidant Enzyme Domain Containing 1 (AAED1), Zinc Finger Protein 367 (ZNF367), U-Box Domain Containing 5 (UBOX5), SET and MYND Domain Containing 1 (SMYDI), Fatty Acid Binding Protein 1 (FABP1), Mitochondrial Ribosomal Protein S27 (MRPS27), and Ring Finger Protein 122 (RNF122). To date, to our knowledge, no reports or obvious evidence have shown that these genes are directly related to various pathways of sex determination. However, they are still worthy of attention because they are located on the sex chromosome, where any variation may have an effect on the genes underlying sex determination. The confirmation of SNP Cyn_Z_6676874 indicated that it was the first barrier regulating sex reversal from genetic females to phenotypic males. We noticed that Z^W individuals may not always reverse into phenotypic male; therefore, we performed GWAS again with only individuals of the Z^W genotype to determine whether a significant gene interacted with the Cyn_Z_6676874 locus. The results revealed no significant locus (Figure 3), possibly because the sample size was limited, or because minor genes control the remaining 17.3% of the genetic variation of sex reversal, and interact with only the Z^W genotype. It is important to increase the sample size of Z^W individuals in order to map all loci, and understand the pathway of sex reversal.

Sex reversal has resulted in considerable increased costs in tongue sole aquaculture in recent years in China, and the uncertainty of the female fry ratio has resulted in panic and risk in investment in aquaculture for farmers; therefore, effective management techniques are necessary to control sex reversal and keep the female ratio high. Usually, a specific pseudomale (ZW/males) ratio was considered to improve female percentage in offspring, because pseudomales carry the W chromosome (Piferet et al. 2012). In our study, we did not find the Z^W/males:X^W/males cross, but the offspring from two Z^W/males:Z^W/males families precluded this hypothesis, and verified its impossibility in aquaculture, similarly to the conclusion of other studies (L. Chen et al. 2014). The sex reversal locus reported here could be used directly in breeding to produce nonreversed half-smooth tongue soles, keeping the phenotypic female ratio of offspring at around one-half. Using Z^W/Z^W males as sires, the genetic female offspring are Z^W, and none of them will reverse into phenotypic males (Figure 4, A and D). If normal female Z^W are used, then the unfavorable allele T will be removed from the population, and the genetic female offspring of the next generations will never reverse into phenotypic males (Figure 4A). These two mating techniques are beneficial for the effective management of the aquaculture of the half-smooth tongue sole.

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