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The Snakeskin Gourami (*Trichopodus pectoralis*) Tends to Exhibit XX/XY Sex Determination

Thitipong Panthum [1,2,3], Nararat Laopichienpong [1,3], Ekaphan Kraichak [4], Worapong Singchat [1,3], Dung Ho My Nguyen [1,2,3], Nattakan Ariyaraphong [1,2,3], Syed Farhan Ahmad [1,3], Narongrit Muangmai [5], Prateep Duengkae [3], Surin Peyachoknagul [1,3], Tariq Ezaz [6] and Kornsorn Srikulnath [1,2,3,7,8,*]

1 Animal Genomics and Bioresource Research Center (AGB Research Center), Faculty of Science, Kasetsart University, 50 Ngamwongwan, Chatuchak, Bangkok 10900, Thailand; thitipong.pa@ku.th (T.P.); nararat.l@ku.th (N.L.); worapong.si@ku.th (W.S.); nguyenhomydung.d@ku.th (D.H.M.N.); nattakan.ari@ku.th (N.A.); syedfarhan.a@ku.th (S.F.A.); fscisrp@ku.ac.th (S.P.)
2 Laboratory of Animal Cytogenetics and Comparative Genomics (ACCG), Department of Genetics, Faculty of Science, Kasetsart University, 50 Ngamwongwan, Chatuchak, Bangkok 10900, Thailand
3 Special Research Unit for Wildlife Genomics (SRUWG), Department of Forest Biology, Faculty of Forestry, Kasetsart University, 50 Ngamwongwan, Chatuchak, Bangkok 10900, Thailand; fforptd@ku.ac.th
4 Department of Botany, Kasetsart University, Bangkok 10900, Thailand; ekaphan.k@ku.th
5 Department of Fishery Biology, Faculty of Fisheries, Kasetsart University, 50 Ngamwongwan, Chatuchak, Bangkok 10900, Thailand; ffisnrm@ku.ac.th
6 Institute for Applied Ecology, University of Canberra, Bruce, ACT 2617, Australia; tariq.ezaz@canberra.edu.au
7 Center of Excellence on Agricultural Biotechnology: (AG-BIO/MHESI), Bangkok 10900, Thailand
8 Amphibian Research Center, Hiroshima University 1-3-1, Kagamiyama, Higashihiroshima 739-8526, Japan
* Correspondence: kornsorn.s@ku.ac.th; Tel.: +66-25625444

Abstract: The snakeskin gourami (*Trichopodus pectoralis*) has a high meat yield and is one of the top five aquaculture freshwater fishes in Thailand. The species is not externally sexually dimorphic, and its sex determination system is unknown. Understanding the sex determination system of this species will contribute to its full-scale commercialization. In this study, a cytogenetic analysis did not reveal any between-sex differences in chromosomal patterns. However, we used genotyping-by-sequencing to identify 4 male-linked loci and 1 female-linked locus, indicating that the snakeskin gourami tends to exhibit an XX/XY sex determination system. However, we did not find any male-specific loci after filtering the loci for a ratio of 100:0 ratio of males:females. This suggests that the putative Y chromosome is young and that the sex determination region is cryptic. This approach provides solid information that can help identify the sex determination mechanism and potential sex determination regions in the snakeskin gourami, allowing further investigation of genetic improvements in the species.

Keywords: single nucleotide polymorphism; recombination; teleost; cryptic sex chromosome; polygenic sex determination; *Trichopodus pectoralis*; sex determination; fluorescence in situ hybridization

Citation: Panthum, T.; Laopichienpong, N.; Kraichak, E.; Singchat, W.; Ho My Nguyen, D.; Ariyaraphong, N.; Ahmad, S.F.; Muangmai, N.; Duengkae, P.; Peyachoknagul, S.; et al. The Snakeskin Gourami (*Trichopodus pectoralis*) Tends to Exhibit XX/XY Sex Determination. *Fishes* **2021**, *6*, 43. https://doi.org/10.3390/fishes6040043

Academic Editor: Michael Møller Hansen

Received: 12 August 2021
Accepted: 17 September 2021
Published: 23 September 2021

1. Introduction

The snakeskin gourami (*Trichopodus pectoralis*, Regan 1910; Anabantoidei: Osphronemidae) is one of the most common air-breathing freshwater fishes in the Indochina Peninsula [1,2]. They can survive in waters with low dissolved oxygen and high organic loads, and are often found in rice paddies, ditches, and streams with dense vegetation [3]. The snakeskin gourami generally feeds on zooplankton, crustaceans, and insect larvae [4]. It is also one of the most popular and colorful species of aquarium fish and an attractive species for commercial farming [5]. The snakeskin gourami has a high meat yield, and traditional dried snakeskin gourami is a popular food [3]. Dried snakeskin gourami is widely consumed in Southeast Asia, and recent developments in this regard have led to increasing

costs of snakeskin gourami in the aquaculture market [6]. The commercial farming of snakeskin gourami has developed rapidly in recent years [5]. Current farming systems face the problem of seed supply limitations, which is exacerbated by the lack of available information on the sex determination system of snakeskin gourami and the unavailability of options for sex control and genetic improvements [7]. A stock assessment during the early life stage is necessary for breeding management; however, the fish progeny take a relatively long time (estimated to be 2–3 years) to reach sexual maturity. Moreover, this species does not show external sexual dimorphism [2], which hinders effective commercial breeding. Mature sex organs can be observed in snakeskin gourami at approximately 1 year of age using ultrasound imaging and evaluation of plasma vitellogenin or sex steroid levels [8]. All of these methods can be performed on adult captive broodstock during the period preceding the first half of the spawning season [9]. Nevertheless, understanding the sex determination system in snakeskin gourami is an important baseline for future research on evolutionary biology, sex development, and genetic improvements in aquaculture. Sex manipulation through gynogenesis or androgenesis in breeding programs is also of considerable economic value [10].

The mechanisms of sex determination in teleosts are extremely diverse and comprise environmental sex determination (ESD), genotypic sex determination (GSD), or a combination of the two [11–13]. For GSD, the sex chromosomes are likely well-differentiated in several amniotes that exhibit male heterogamety (XX/XY), female heterogamety (ZW/ZZ), or multiple sex chromosomes that determine sex; however, most teleosts do not show heteromorphic sex chromosomes [14,15]. A comparison of sex ratios and gonad morphology in snakeskin gourami revealed a tendency towards GSD [16], but there is little evidence concerning its sex determination system. The osphronemids comprise 15 genera and 46 species. Of these, most species show highly conserved karyotypes with 2n = 46 (composed of only acrocentric chromosomes), some exhibit GSD, and others exhibit temperature-dependent sex determination [17,18]. Based on the phylogenetic mapping of homomorphic and heteromorphic patterns of sex chromosomes in this lineage, we hypothesized that snakeskin gourami exhibits a ZZ/ZW system (Figure 1). Similar trends of diverse and rapid evolution of sex chromosomes were previously reported in closely related teleost species [11,14,15,18–20], suggesting that sex chromosomes and sex determination systems have evolved independently in different lineages. The investigation of sex chromosomes and sex-determining mechanisms is, therefore, a prerequisite for the development of an accurate method of sex identification. Several techniques were employed to identify sex chromosomes. Karyotype analysis is a simple and conventional cytogenetic approach to examine heteromorphic sex chromosomes in teleosts and other animals [21–29]. However, this method is not suitable for species with homomorphic sex chromosomes, even though several sex chromosomes show a high accumulation of repetitive elements [25,30–34]. In recent years, next-generation sequencing technologies have facilitated the discovery of numerous genetic markers in almost any organism at an affordable cost, allowing the investigation of genetic diversity within and between populations [35]. Only a fraction of the homologous regions in the genome of non-model species can be sequenced and genotyped for single nucleotide polymorphisms (SNPs) to identify the genomic regions of sex-determining loci [19,20,36,37]. Diversity Arrays Technology (DArTseq), a technique developed by Diversity Arrays Technology Pty Ltd. (Canberra, Australia), generates restriction site-specific presence-absence (PA) markers and is an effective method for identifying sex-linked loci using SNP loci.

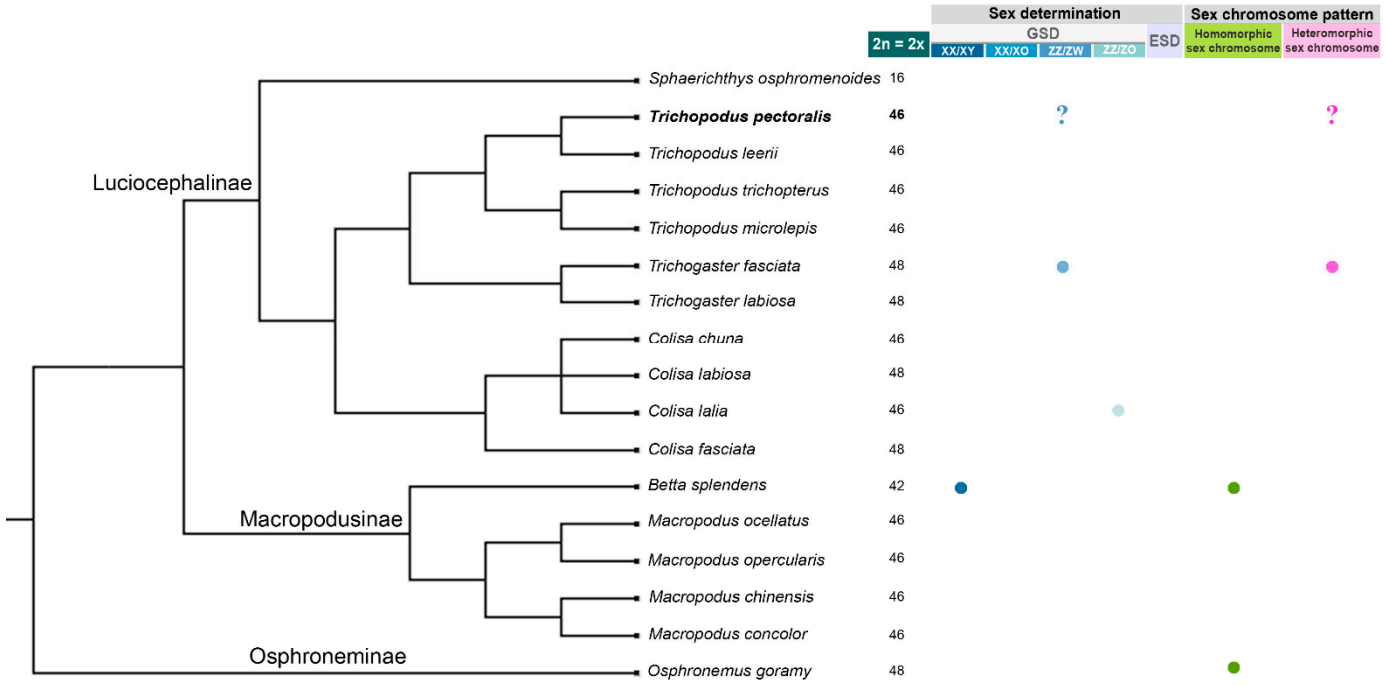

Figure 1. Schematic representation showing variations in chromosome numbers and sex determination method among different species in family Osphronemidae. The phylogeny was partially derived from Ruber et al. [38]. The chromosome numbers and sex determination systems of *Sphaerichthys osphromenoides*, *Trichopodus pectoralis*, *T. leerii*, *T. trichopterus*, *T. microlepis*, *Colisa chuna*, *C. labiosa*, *C. lalia*, *C. fasciata*, and *Osphronemus goramy* were obtained from Koref-Santibanez and Paepke [39]. The chromosome numbers of *Trichogaster fasciata* and *Trichogaster labiosa* were obtained from Manna and Prasad [40], that of *Betta splendens* was obtained from Grazyna et al. [41], those of *Macropodus ocellatus*, *M. opercularis*, and *M. concolor* were obtained from Koref-Santibanez et al. [42], and that of *M. chinensis* was obtained from Jianxun et al. [43]. The bullet symbols indicate a presence data. The question mark symbols indicate a hypothesis. Lack of symbol indicate an absence of data.

Here, we address the aforementioned hypothesis using conventional and molecular cytogenetic approaches. We also performed DArTseq to identify a number of novel SNP/PA loci in captive-bred individuals with known phenotypic sex assignment and to determine the potential sex-determining system and associated regions in snakeskin gourami. Additionally, the mapped DArTseq sequences were used to search for homologies with other model teleosts (the Japanese rice fish: *Oryzias latipes*, zebrafish: *Danio rerio*, and the Japanese puffer: *Takifugu rubripes*) and vertebrates (chicken: *Gallus gallus*) using comparative genomic analyses. Our findings provide novel insights into the evolutionary history of sex chromosomes in teleosts and other vertebrates.

2. Materials and Methods

2.1. Specimens and DNA Extraction

Ten male and ten female snakeskin gourami individuals from several clutches were donated by the Ayutthaya Farm (Ayutthaya, Thailand). The sample individuals were adults with a standard weight of 20–50 g and a length of 12–15 cm. Sex was determined based on examining internal genital anatomy [44]. After each fish had been sacrificed by severing the spinal cord anterior to the dorsal fin, the fins were collected for DNA extraction, the kidneys and the gills for mitotic chromosome preparation, and the testes for meiotic chromosome preparation. Molecular identification of the species was performed according to Kitano et al. [45], Wyneken et al. [46], Hubert et al. [47] and Rüber et al. [48], and all experimental procedures were approved (approval no. ACKU63-SCI-010) by the Animal Experiment Committee of Kasetsart University and conducted in accordance with the Regulations on Animal Experiments at Kasetsart University. Genomic DNA

was extracted following the standard salting-out protocol with slight modifications for different tissues, as described previously [49]. The high-molecular-weight DNA samples were stored at −20 °C until they were used for DArTseq library construction, as described previously [19,20,37,50].

2.2. Development of DArTseq Arrays

A detailed description of the DArTseq methodology can be found in Jaccoud et al. (2001) [51]. Multiple loci were genotyped using Diversity Arrays Technology Pty Ltd. (DArTseq, Canberra, ACT, Australia), to identify the SNP loci and in silico DArT markers (also called the PA markers, as any variability in the SNP loci generates presence/absence (PA) polymorphisms in restriction sites). These data were used to determine candidate sex-specific loci in male and female individuals. Approximately 100 ng of DNA was used from each sample to develop the DArTseq arrays. The DNA samples were subjected to digestion and ligation reactions, as described previously [19,20,37,50,52] and were digested with the restriction enzymes *Pst*I and *Sph*I. Ligation reactions were performed using two adaptors: a *Pst*I-compatible adaptor consisting of an Illumina flow-cell attachment sequence, primer sequence, and a unique barcode sequence; and an *Sph*I-compatible adaptor consisting of an Illumina flow-cell attachment region. The ligated fragments were PCR-amplified by the initial denaturation at 94 °C for 1 min, followed by 30 cycles of 94 °C for 20 s, 58 °C for 30 s, and 72 °C for 45 s, with a final extension step at 72 °C for 7 min. Equimolar amounts of amplification products from each individual were pooled and subjected to Illumina's proprietary cBot (http://www.illumina.com/products/cbot.html, accessed on 15 August 2020) Bridge PCR, followed by sequencing on the Illumina HiSeq 2000 platform. Single-read sequencing was performed for 77 cycles and the sequences were processed using proprietary DArTseq analytical pipelines [53]. The outputs generated by DArTsoft14 were filtered based on reproducibility values, the average count for each sequence (sequencing depth), the average SNPs in each allele, and call rate (proportion of samples for which the marker was scored), as described previously [19,20,37,50]. Sex-specific or sex-linked loci were obtained from the SNPs and PA marker analyses as follows. For loci with an XX/XY sex determination system, the SNPs and PA loci sequenced in 70%, 80%, 90%, and 100% of males included in a separate data set. The SNP and PA loci were filtered using different proportions of males and females. Loci that passed the 100% filtering criterion were designated as perfectly sex-linked or sex-specific, whereas those passing at 70–90% were considered moderately sex-linked loci, as described previously [19,20,37,50]. Equivalent modifications of this approach were used to target loci with a ZZ/ZW system. The Hamming distance was calculated to determine the number of common loci between male and female individuals, so as to calculate pairwise differences in SNP and PA loci using the "rdist" function in the "rdist" package in R version 3.6.2. The Hamming distance, Cochran–Armitage trend test (CATT), and polymorphism information content (PIC) were used as indices to evaluate the informativeness of the calculated SNP and PA loci, as described previously [19,20,37,50,54,55]. The probability of candidate sex-linked and sex-specific loci showing random associations with sex in a small sample size was estimated using the formula $P_i = 0.5^n$, where P is the probability for a given locus, i is sex-linked, 0.5 is the probability that either a female is homozygous or a male is heterozygous at a given locus, and n is the number of individuals sequenced at the locus, as described previously [19,20,37].

2.3. Comparison of Potential Sex-Linked Loci

Significant differences among the three groups of moderately sex-linked loci (90:10, 80:20, and 70:30) were analyzed using the chi-square test and Kruskal–Wallis test for PA loci and the Nemenyi test for SNP loci, as implemented in the PMCMR package in R [54]. These analyses were based on the mean heterozygosity and standard deviation of the loci. All candidate loci were plotted for each individual using the "glPlot" function in the "dartR"

R package. A principal coordinate analysis based on all groups of moderately sex-linked loci was used to visualize the relatedness between males and females [19,20,37,50,54,56].

2.4. Homology Searching

All sex-linked loci that met our criteria and showed a statistically significant association with the known sex phenotype were BLAST-searched within the National Center for Biotechnology Information (NCBI) database. The results were used to investigate the homologies between the sex-linked SNP/PA loci and the available reference genomes of teleosts, including those of the Japanese rice fish, *O. latipes* (accession no. GCF_002234675.1) [57]; the zebrafish, *D. rerio* (accession no. GCA_000002035.4) [58]; the Japanese puffer fish, *T. rubripes* (accession no. GCA_901000725.2) [59]; and the chicken: *G. gallus* (accession no. AADN00000000.5) [60]. Using the BLASTn program, all loci were then used to search the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 30 August 2020)) and RepBase version 19.11 [61] (Genetic Information Research Institute, http://www.girinst.org/repbase/, accessed on 30 August 2020), which is a specialized database of repeated or other significant sequences that only reports results with *E*-values < 0.005 and query coverage > 55% similarity [19,20,37,50].

2.5. Chromosome Preparations and Mapping the Chromosomal Locations of Microsatellite Repeat Motifs, Telomeric (TTAGGG)$_n$ Sequences, and Major Ribosomal RNA Genes with Fluorescence In Situ Hybridization

Mitotic chromosome spreads were prepared using the air-drying method described by Suntronpong et al. [62]. The chromosomal locations of telomeric $(TTAGGG)_n$ sequences, 18S–28S ribosomal RNA (rRNA) genes, and 19 microsatellite repeat motifs, $(CA)_{15}$, $(GC)_{15}$, $(GA)_{15}$, $(AT)_{15}$, $(CAA)_{10}$, $(CAG)_{10}$, $(CAT)_{10}$, $(CGG)_{10}$, $(GAG)_{10}$, $(AAT)_{10}$, $(AAGG)_8$, $(AATC)_8$, $(AGAT)_8$, $(ACGC)_8$, $(AAAT)_8$, $(AAAC)_8$, $(AATG)_8$, $(AAATC)_6$, and $(AAAAT)_6$, were determined using fluorescence in situ hybridization (FISH) as described previously [63–66]. The FISH signals were captured using a cooled charge-coupled device camera mounted on a Nikon Eclipse 80 microscope and processed using the NIS-Elements BR 3.2 software (Nikon Corporation, Tokyo, Japan).

3. Results

3.1. Identification of the Sex Determination System and Sex-Linked Loci in the Snakeskin Gourami

Of the samples from 20 individuals, 4 did not produce sufficient read depth for an analysis. In total, we sequenced 2925 SNP loci and 3703 PA loci from a sample size of 16 individuals (seven males and nine females). PIC values ranged from 0.1 to 0.5 with an average of 0.32 from all loci. The overall distribution of PIC values was asymmetrical and skewed toward higher values. To determine whether the sex determination system in the snakeskin gourami was GSD (XX/XY or ZZ/ZW) or ESD, we compared the number of SNP and PA loci after filtering by using a set of criteria with gradual changes. For the ZZ/ZW system, after filtering using the criterion of 30:70 ratio of males:females, we obtained 12 SNPs and 66 PA loci that were female-linked. The Hamming distance, measured using sex-linked SNP and PA loci under the null exclusive model, showed within-sex distances of 0.618 ± 0.038 in males and 0.183 ± 0.008 in females for SNP loci, and 0.667 ± 0.026 in males and 0.382 ± 0.029 in females for PA loci. Between-sex distances were 0.714 ± 0.022 for SNP loci and 0.685 ± 0.027 for PA loci. A CATT analysis verified that phenotype was significantly associated with 5 SNP loci (χ^2 = 4.00–11.00, $p < 0.001$) and 59 PA loci (χ^2 = 3.00–10.00, $p < 0.001$). However, after filtering with 20:80, 10:90, and 0:100 ratios, we did not find any significant sex-specific or -linked SNP/PA loci.

For the XX/XY system, filtering for a 70:30 ratio of males:females yielded 36 SNP loci and 19 PA loci that were male-linked. The Hamming distances between male and female snakeskin gourami were calculated using sex-linked SNP and PA loci, and showed within-sex distances of 0.423 ± 0.017 in males and 0.573 ± 0.012 in females for SNP loci, and 0.429 ± 0.02 in males and 0.354 ± 0.016 in females for PA loci. The between-sex distances were 0.745 ± 0.009 for SNP loci and 0.675 ± 0.012 for PA loci. The within-sex distances

were lower than the between-sex distances. A CATT analysis was used to verify significant associations of phenotypic sex with 22 SNP loci (χ^2 = 3.00–12.00, $p < 0.001$) and 17 PA loci (χ^2 = 3.00–13.00, $p < 0.001$). In total, of 3 SNP loci and 3 PA loci were associated with males based on the filtering criterion of 80:20 ratio of males:females. Using the sex-linked SNP and PA loci, we calculated the proportional pairwise Hamming distances between male and female snakeskin gourami, and these results showed within-sex distances of 0.286 ± 0.07 in males and 0.582 ± 0.05 in females for SNP loci and 0.286 ± 0.101 in males and 0.0 ± 0.0 in females for PA loci (Figures 2 and 3). The between-sex distances were 0.815 ± 0.028 for SNPs and 0.857 ± 0.044 PA loci. A CATT analysis verified a significant association of phenotypic sex with 3 SNP loci (χ^2 = 8.00, $p < 0.001$) and 3 PA loci (χ^2 = 12.00–30.00, $p < 0.001$). However, after filtering with 90:10 and 100:0, we did not find any significantly sex-specific or -linked SNP/PA loci (Figure 3). Chi-square tests showed that the 70:30 and 80:20 filtering criteria indicated no significant differences in males ($\chi^2 = 1.0383 \times 10^{-31}$, $p = 1$) and females ($\chi^2 = 3.7886 \times 10^{-32}$, $p = 1$) for PA loci. Moreover, Kruskal–Wallis tests showed that these filtering criteria produced no significantly different percentages of heterozygosity in males (H = 2.27, p = 0.132) and females (H = 0.734, p = 0.392) for SNPs (Figure 4). Pairwise comparisons using the Nemenyi test with chi-squared approximation for independent samples also revealed that the 70:30 and 80:20 filters produced no significantly different heterozygosity compared with other filters in males (p = 0.13) and females (p = 0.39) (Figure 4). A principal coordinates analysis plot revealed that the grouping was more similar between the sexes (Figure 5).

3.2. Random Sex-Linkage Estimation

Across a range of sample sizes and loci, samples were required from 16 individuals of the snakeskin gourami (7 males and 9 females) to minimize the probability of selecting less than one spurious sex-linked marker. For the 16 specimens, the probability of a single locus exhibiting a sex-linked pattern by chance (i.e., *P*i) was 1.526×10^{-5} based on 6628 loci (including SNP and PA loci), whereas the expected sex linkage was estimated to be 0.1011. Therefore, the random sex-linked markers in the snakeskin gourami were higher than the expected values in this study.

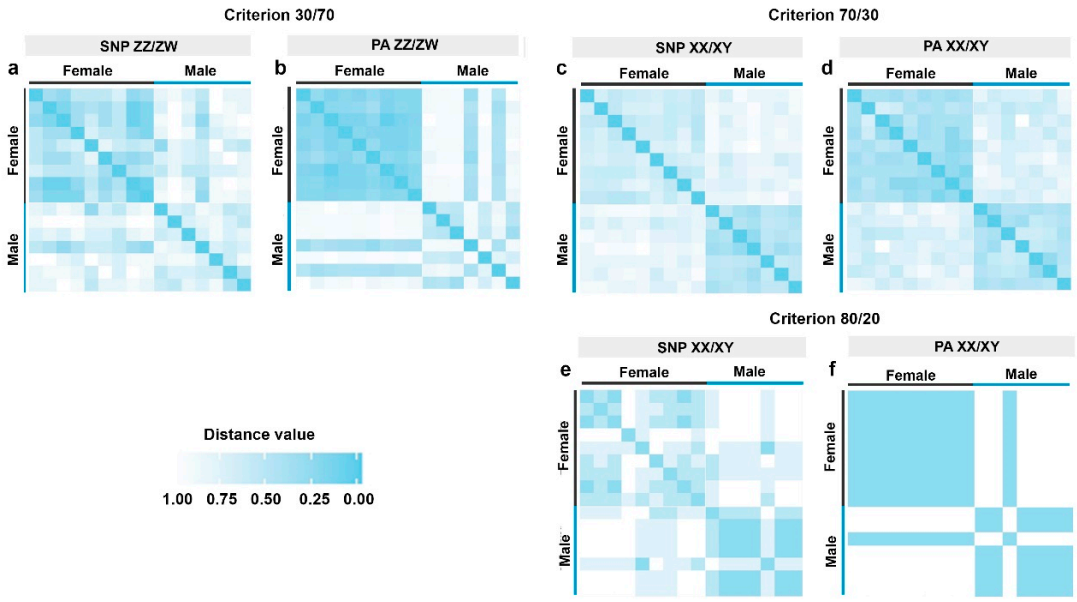

Figure 2. Hamming distances between male and female snakeskin gourami (*Trichopodus pectoralis*) using single nucleotide polymorphism (SNP) and presence-absence (PA) loci. (**a**) SNP loci filtered with the criterion of 30:70 (males:females), (**b**) PA loci filtered for 30:70 (males:females) for ZZ/ZW system, (**c**) SNP loci filtered for 70:30 (males:females), (**d**) PA loci filtered for 70:30 (males:females), (**e**) SNP loci filtered for 80:20 (males:females), and (**f**) PA loci filtered for 80:20 (males:females) for XX/XY system.

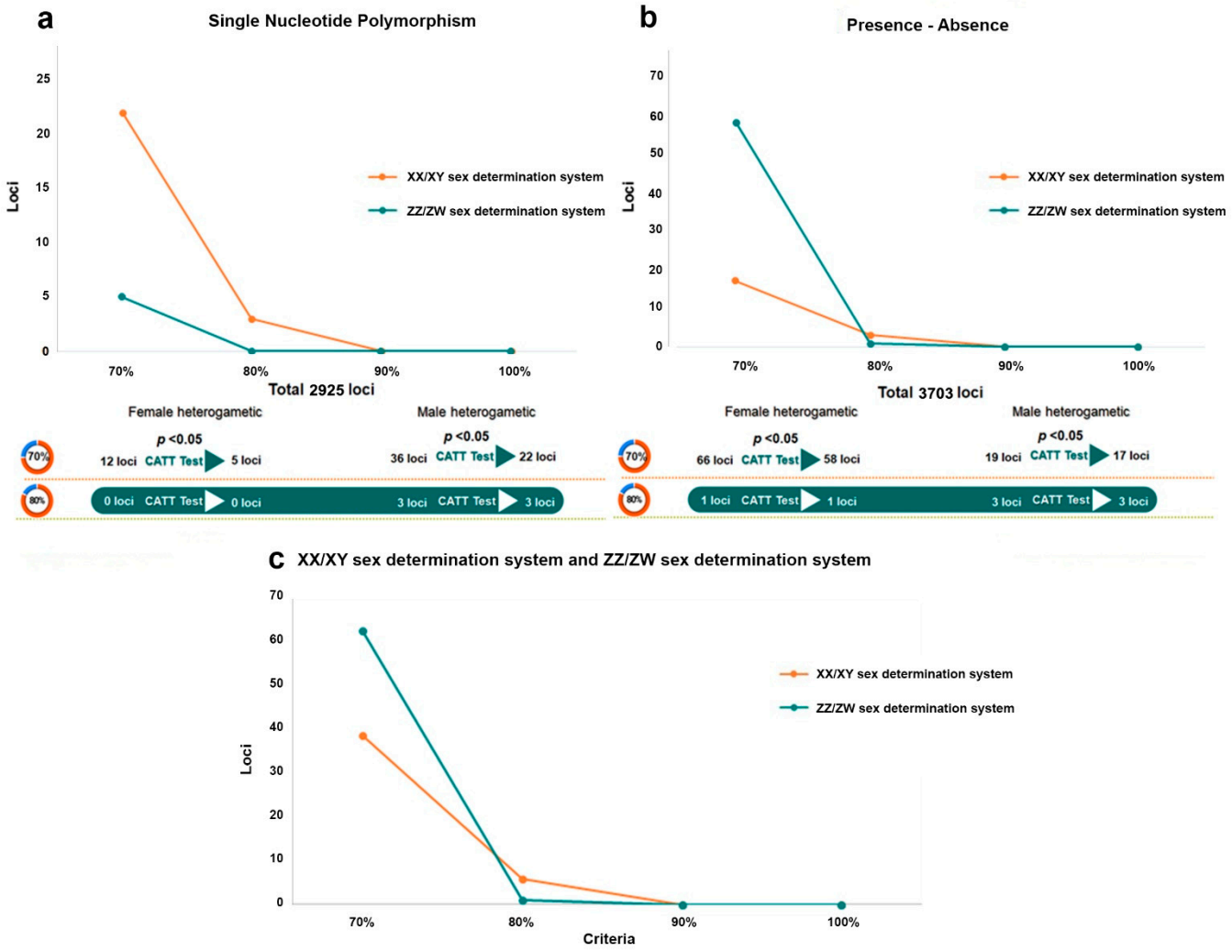

Figure 3. Line charts displaying threshold of single nucleotide polymorphism (SNP) and presence-absence (PA) loci. (**a**) SNP loci filtered for 70:30, 80:20, 90:10 and 100:0 ratios of males:females, (**b**) PA loci filtered for 70:30, 80:20, 90:10 and 100:0 (males:females), and (**c**) comparison of ZW and XY sex determination systems using loci filtered for 70:30, 80:20, 90:10 and 100:0 ratios of males:females.

3.3. Homology of Putative Sex-Linked Loci

Sex-linked loci in male snakeskin gourami had sequence homology with the genomes of the Japanese rice fish, *O. latipes*; zebrafish, *D. rerio*; the Japanese puffer fish, *T. rubripes;* and the chicken: *G. gallus*. No substantial differences were observed between the linkage groups. Therefore, all these loci may be located on the same chromosome. Based on the global BLAST analyses against the NCBI databases, 9 of the 39 SNP/PA loci were homologous with putative genes: *CFAP92* (*E*-value 0.047 and similarity 79.49%), *ZIC3* (*E*-value 0.05 and similarity 78.38%), *IFT88* (*E*-value 0.015 and similarity 79.49%), *IL4* (*E*-value 0.15 and similarity 80.49%), *IL13* (*E*-value 0.15 and similarity 80.49%), *XIRP2* (*E*-value 0.15 and similarity 80.00%), *PATJ* (*E*-value 1×10^{-16} and similarity 90.62%), *NEB* (*E*-value 1×10^{-16} and similarity 90.62%), *PLA2R1* (*E*-value 2×10^{-8} and similarity 94.87%), and *NEMF* (*E*-value 0.016 and similarity 80.56%) (Table S1). Moreover, 10 SNP/PA loci showed partial homology with transposable elements, including three long terminal repeat retrotransposons of *Gypsy* families (similarity 80–100%), two hAT DNA transposons (similarity 78%), and one endogenous retrovirus (similarity 80%) (Figure S1).

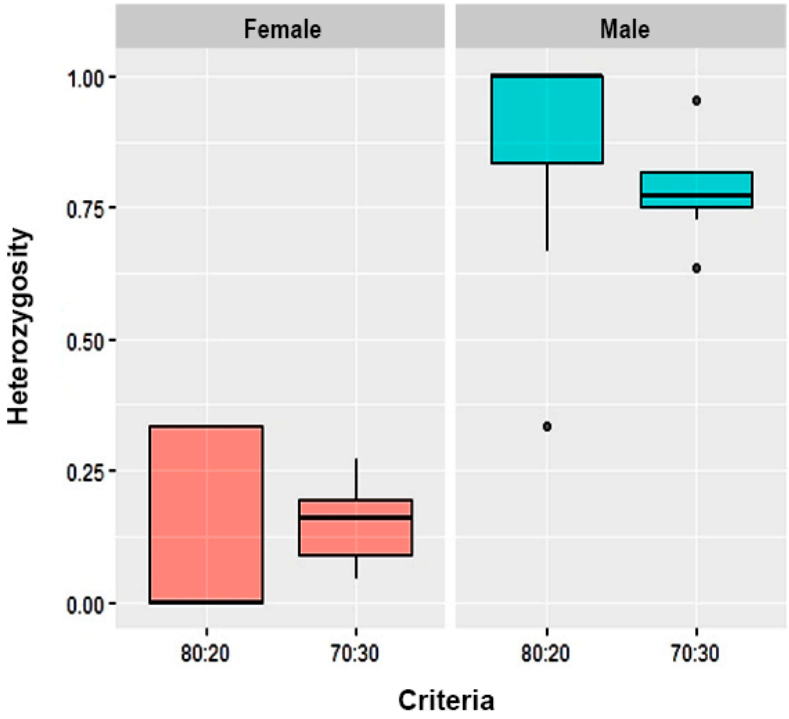

Figure 4. Kruskal–Wallis tests of filtering criteria showed no significantly different percentages of heterozygosity in males (H = 2.27, p = 0.132) and females (H = 0.734, p = 0.392) for single-nucleotide polymorphic (SNP) of the XX/XY sex determination system. The bullet points indicate an outside median ± interquartile range (IQR: Q3–Q1).

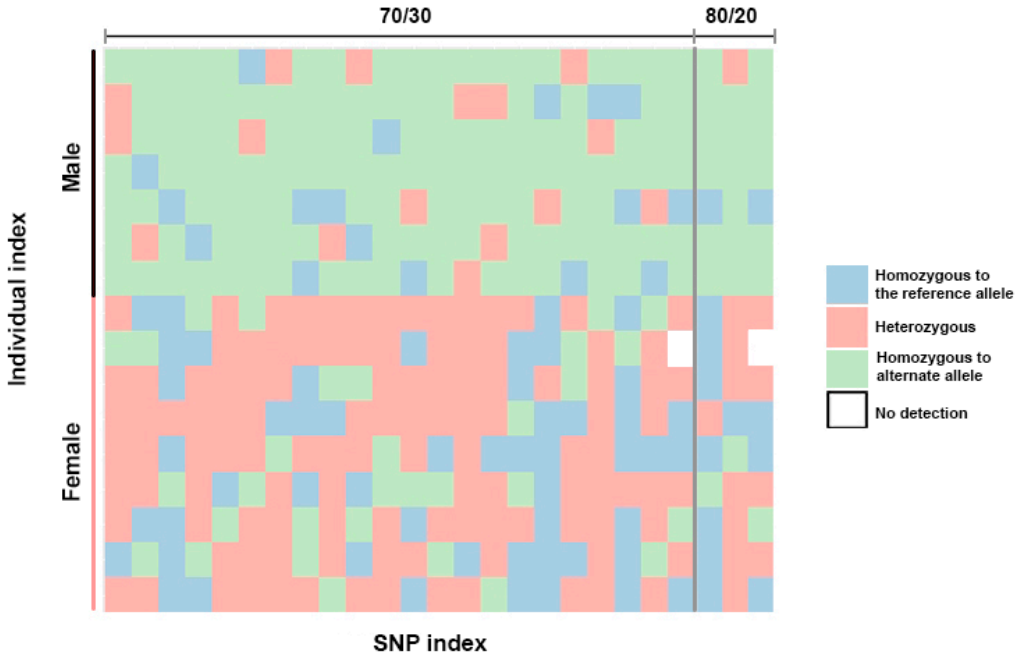

Figure 5. An index of the 16 moderately sex-linked loci with criterion of 70:30 (male:female) (XX/XY sex determination system) created using the "glPlot" function in the R package, "dartR". Blue indicates homozygosity to the reference allele, pink indicates heterozygosity, and green indicates homozygosity to the allele containing the alternate SNP.

3.4. Karyotype and Meiotic Configuration

More than 20 Giemsa-stained metaphase spreads were examined for each snakeskin gourami individual. The diploid chromosome number was 46, including acrocentric chromosomes (Figure S2). The chromosomes were arranged sequentially based on size, and chromosome pairs could not be identified by size. Light microscopy of the meiotic configuration in the spermatocytes of male snakeskin gourami showed normal stages of meiosis (Figure S3).

3.5. Chromosomal Locations of the 18S–28S rRNA Genes, Telomeric $(TTAGGG)_n$ Sequences, and Microsatellite Repeat Motifs

FISH signals for the 18S–28S rRNA genes were detected in the pericentromeric region of one chromosome pair. Hybridization signals of TTAGGG repeats were observed at the telomeric ends of all chromosomes, but no interstitial signals were found (Figure 6). Moreover, no signals were detected for the 19 microsatellite repeat motifs: $(CA)_{15}$, $(GC)_{15}$, $(GA)_{15}$, $(AT)_{15}$, $(CAA)_{10}$, $(CAG)_{10}$, $(CGG)_{10}$,$(CAT)_{10}$, $(GAG)_{10}$, $(AAT)_{10}$, $(ACGC)_8$, $(AAGG)_8$, $(AATC)_8$, $(AAAC)_8$, $(AATG)_8$, $(AAAT)_8$, $(AGAT)_8$, $(AAATC)_6$, and $(AAAAT)_6$.

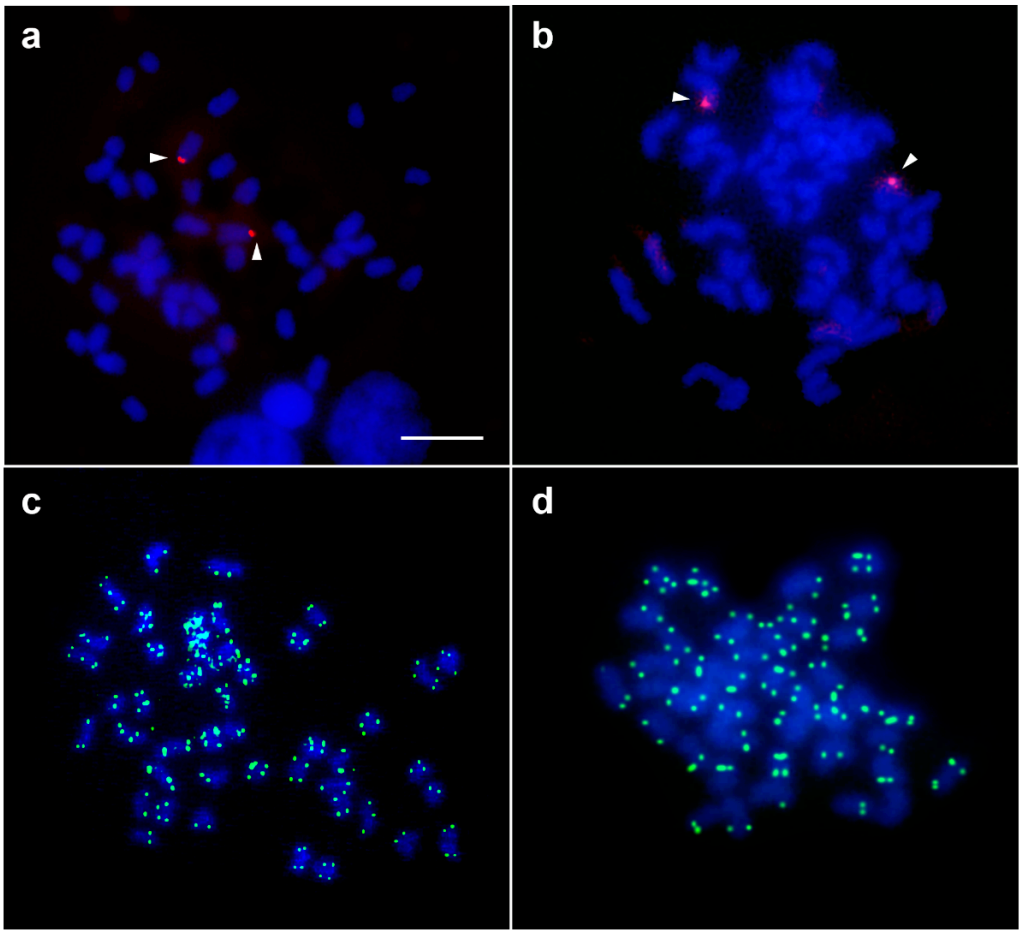

Figure 6. Chromosomal locations of 18S rRNA genes in (**a**) males and (**b**) females, and telomeric $(TTAGGG)_n$ sequences in (**c**) males and (**d**) females of the snakeskin gourami (*Trichopodus pectoralis*). Arrowheads indicate hybridization signals. The scale bar represents 10 μm.

4. Discussion

Snakeskin gourami is one of the top five freshwater fish used in aquaculture in Thailand. Many hatcheries import their broodstock from grow-out farms and manipulate, culture, and maintain fish commercially. The farming of snakeskin gourami offers an attractive income and good commercial prices [67]. Many teleosts exhibit substantial sexual

dimorphism in growth rate and body size, which are closely linked to multiple economically important traits. Accurate sex identification of the snakeskin gourami based on sexual size dimorphism is of considerable value to fish farmers for the use of sex-controlled breeding biotechnologies [68]. Here, we used 20 individuals (10 males and 10 females), of which 16 (7 males and 9 females) were successfully used to identify genome-wide SNP/PA loci. This allowed us to predict sex-linked loci for snakeskin gourami. The probability of a single locus spuriously showing a sex linkage was 0.1026. Although this is a relatively small sample size, we sampled four sex-linked loci, and the identification of any sex-linked loci by chance was unlikely. Several similar cases with small sample sizes (<5 individuals per sex) have also been reported in gecko lizards, pythons, and North American green frogs (*Rana clamitans*) [69,70].

Gynogenesis primarily makes use of heterogeneous or inactivated sperm to activate eggs, which are then heat-shocked to prevent the second meiotic division. This process essentially induces female self-fertilization [71]. There have been several studies on gynogenesis and sex manipulation in snakeskin gourami [72–74] that enabled us to predict the XX/XY sex determination system in this species. However, this is phylogenetically different from the usual sex determination systems of osphronemids (Figure 1). Here, we used karyotyping and molecular cytogenetic (18S–28S rRNA genes, telomeric repeats, and microsatellite repeat motifs) analyses to show similar patterns between males and females. We did not find any signals of chromosome-specific microsatellite amplification, such as those found in chromosome Y (or W) with the set of probes used here. The same set of microsatellite repeat motifs was used to perform chromosome mapping in many vertebrates showing large blocks of hybridization signals on sex chromosomes [31,62,75]. We also did not detect any diakinesis-meiotic (I) cells with partially paired bivalents (speculated to be heteromorphic X and Y chromosomes) or meiotic (II) cells with condensed chromosomes (speculated to be the Y chromosome) (Figure S3). However, our meiotic configuration result did not allow us to make a definitive conclusion about the presence/absence of sexual bivalence because we were unable to identify the asynaptic ends of sexual bivalence under a synapsis analysis [76]. Comparative genomic hybridization permitting the detection of chromosomal copy number changes or sex-specific region detection on low-quality chromosomes is also required to assist the localization of potential sex-specific regions [77]. However, our study successfully identified 39 SNP/PA male-linked loci, and 1 female-linked locus. This suggests that snakeskin gourami tends to exhibit an XX/XY sex-determining system. Male-beneficial mutations arising close to the sex locus on the Y chromosome are strongly favored in males, and should also drive a progressive arrest of recombination in males to enhance sex linkage [78,79]. This suggests that the Y chromosomes of snakeskin gourami are very young sex chromosomes. The emergence of an XY sex determination system is likely to be a hallmark of evolutionary autapomorphy compared with giant gourami (*Trichogaster fasciata*), or dwarf gourami (*Colisa lalia*) which have a ZZ/ZW sex determination system [80] (Figure 1). We also did not find any male-specific SNP/PA loci indicating distinct male-specific regions of the Y chromosome. By contrast, the male-linked loci indicated either partial recombination between the X and Y sex chromosomes of snakeskin gourami. This suggests that the non-recombinant regions of the Y chromosome might be too cryptic for detection by the DArTseq methodology. Moreover, it is possible that the noise from high-throughput sequencing and random biological variation/association, especially with relatively small sample sizes, can be used to identify sex-linked loci outside the sex determination region or even in autosomes [81–84]. However, we observed a female-linked locus, which were occasionally observed in the same individual but with different linkage groups. This may be caused by recombination events that occur frequently in regions of homomorphic sex chromosomes, and heterozygous sex-linked loci in females may not always indicate female heterogametic sex determination [85]. Alternatively, several genes might regulate the sex determination system in a species in a system known as polygenic sex determination (PSD), observed across teleosts [86]. In several teleost species, sex is determined by either several loci dispersed throughout the

genome, or by multiple allele combinations located on a specific pair of chromosomes, as observed in zebrafish, Lake Malawi cichlid fish [87] and the European sea bass [88]. PSD may appear by the modification of existing sex chromosomes to create a third functional sex chromosome at the same locus, or by the modification of autosomal loci in other regions of the genome to create novel inputs for gonadal development regulations [89].

A total of 10.26% SNP/PA loci that were male-linked showed homology with genes encoding amino acids. Some of these, including *ZIC3*, *IFT88*, *IL13*, and *CFAP92*, are involved in sex determination and sex development pathways in vertebrates [90–93] (Table S2). In addition, 25.64% of the SNP/PA loci were homologous to transposable elements. These are often distributed on sex chromosomes in the medaka (*O. latipes*), platyfish (*Xiphophorus maculatus*), guppy (*Poecilia reticulata*), and tilapia (*Oreochromis niloticus*) [81–84]. Transposable element activity may play an important role in sex chromosome differentiation through the mechanisms of chromosomal breakage, deletion, and rearrangement [94,95]. Transposable elements in regions that experience recombination may regulate the gene expression pattern of the Y sex chromosome by interrupting the gene structure, resulting in gradual silencing and degeneration of the chromosome [96]. These male-linked loci, rather than female-linked loci, may support the notion of an XX/XY sex determination system in the snakeskin gourami. By contrast, homology searches of the male-linked loci revealed that two of the four male-linked SNP/PA loci showed homology with partial sex chromosomal linkages in amniotes (Tables S1–S4), whereas no female-linked SNP/PA loci were matched. Genomic convergence has also been detected by comparative genomic studies in which unrelated sex chromosomes share sex chromosomal linkage homologies across distantly related species [19,20,24–28,30,50,64,96–98]. It is likely that the convergent evolution of sex chromosomes across distantly related taxa has led to genomic elements that are particularly efficient in sex determination [99]. Further evidence, such as whole-genome sequencing or molecular combing, is required to identify this linkage homology.

The most parsimonious explanation of the results supports the hypothesis from a previous study, i.e., diploid gynogenesis in snakeskin gourami produces all females, which suggests the tendency for an XX/XY sex determination system in this species. These studies suggest that sex-linked, X chromosome-linked, and Y chromosome-linked genetic markers are very important in species that lack distinguishable sexually dimorphic phenotypes and in specimens at early stages of development that lack secondary sex characteristics [100,101]. However, none of the 6 male-linked loci discovered independently in the snakeskin gourami were successfully validated. A non-specific band was detected in a few female individuals, possibly due to the stability of the primer binding site. These markers failed the validation step, and were not included in further analyses (data not shown). The PCR validation step often fails when using a DArTseq or RADseq bioinformatics analysis [50,70,102]. This may be because of conserved regions adjacent to the sex-specific restriction sites in both sexes, particularly in case where the non-recombining portion of the Y or W chromosome is extremely small [103]. Biases in the DArTseq method are related to GC content, library preparation, and other factors that may limit success [104], and success depends on sampling an adequate number of individuals from each sex with sufficient read depth [105]. A PCR-based approach for sex identification is required as a genotypic tool for the practical sexing of individuals in populations. Future research may also focus on polymerase chain reaction-restriction fragment length polymorphism or melting curve analysis, which offers more sensitive detection [106]. Analyses of additional snakeskin gourami specimens from different populations are required to examine the presence of markers across populations.

5. Conclusions

This study evaluated genome-wide SNPs in snakeskin gourami using the DArTseq methodology. Considering the small portion of the genome sequence, we identified a considerable number of male-linked loci. The results indicated that snakeskin gourami tended to have an XX/XY sex determination system. However, it remains unclear whether

the large genomic regions between X-specific and Y-specific fragments are associated with sex chromosome differentiation and sex-determining regions. Further analysis of unrelated individuals did not reveal population-wide association with phenotypic sex assignment, but indicated that heterozygosity is relatively common at both loci in larger and more wide-spread populations. Thus, these are likely to be useful loci for the development of sexing techniques for managing pedigrees, sex-controlled breeding programs, and genetic improvements. The control of sex and reproduction has been the primary facilitator of large-scale global industrial aquaculture programs. A high-quality complete genome assembly of the snakeskin gourami is required to further elucidate its mechanism of sex determination, ultimately leading to genetic improvements of this promising aquaculture species. This approach will also provide a solid background for further studies to identify the mechanisms of sex determination and potential sex-determining regions in teleosts.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/fishes6040043/s1, Figure S1: Transposable element searching from SNP and PA loci of snakeskin gourami (*Trichopodus pectoralis*). The results show three long terminal repeat retrotransposons in the Gypsy family, two hAT DNA transposons, and one endogenous retrovirus (ERV1), Figure S2: Giemsa-stained karyotypes of (a) male and (b) female snakeskin gourami (*Trichopodus pectoralis*). The scale bar represents 10 µm, Figure S3: Meiotic cell division of the snakeskin gourami (*Trichopodus pectoralis*), including the (a) interphase and the (b) leptotene, (c) pachytene, (d) diplotene phases. The scale bar represents 10 µm, Table S1: Gene function and pathway for the SNP and PA loci of the snakeskin gourami (*Trichopodus pectoralis*); 70:30 (males:females), Table S2: Homologies of the SNP and PA loci in the snakeskin gourami (*Trichopodus pectoralis*); 70:30 (males:females), Table S3: Chromosomal locations of the SNP and PA loci in the snakeskin gourami (*Trichopodus pectoralis*), as determined by BLAST analysis with the genomes of the Japanese rice fish (*Oryzias latipes*), zebrafish (*Danio rerio*), Japanese puffer fish (*Takifugu rubripes*), and chicken (*Gallus gallus*); 70:30 (male:female), Table S4: Chromosomal locations of the SNP and PA loci in the snakeskin gourami (*Trichopodus pectoralis*), as determined by BLAST analysis against the Japanese rice fish (*Oryzias latipes*), zebrafish (*Danio rerio*), Japanese puffer fish (*Takifugu rubripes*), and chicken (*Gallus gallus*) genomes; 80:20 (male:female).

Author Contributions: Conceptualization: T.P. and K.S.; methodology, T.P., N.L., E.K. and K.S.; project administration: K.S.; Writing—original draft: T.P. and K.S.; Writing—review and editing: T.P., N.L., E.K., W.S., D.H.M.N., N.A., S.F.A., N.M., P.D., S.P., T.E. and K.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was financially supported in part by the Graduate Scholarship Program of the Graduate School, Kasetsart University, Thailand awarded to T.P. and K.S., the Office of the Ministry of Higher Education, Science, Research and Innovation; and the Thailand Science Research and Innovation through the Kasetsart University Reinventing University Program 2021 awarded to T.P. and K.S., the Royal Golden Jubilee Ph.D. program under the Thailand Research Fund (TRF) awarded to D.H.M.N. and K.S. (No. PHD01392561), the Thailand Research Fund (TRF) (No. RSA6180075) provided through a grant awarded to K.S., a Postdoctoral Researcher award at Kasetsart University awarded to S.F.A. and K.S., and International SciKU Branding (ISB), Faculty of Science, Kasetsart University awarded funds to K.S. No funding source was involved in the study design, collection, analysis and interpretation of the data, writing the report and the decision to submit the article for publication.

Institutional Review Board Statement: Animal care and all experimental procedures were approved by the Animal Experiment Committee at Kasetsart University, Thailand (Approval No. ACKU63-SCI-010) and conducted in accordance with the Regulations on Animal Experiments at Kasetsart University.

Data Availability Statement: The full data set and metadata from this publication are available from the Dryad Digital Repository. Data set, https://doi.org/10.5061/dryad.tdz08kpx1 (accessed on 21 August 2020).

Acknowledgments: We thank Sompon Muangthong and Bundit Ruttanasatian (Ayutthaya Farm, Ayutthaya, Thailand) for all snakeskin gourami specimens.

Conflicts of Interest: The authors declare no conflict of interest.

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