THESIS

AN INVESTIGATION OF ZZ/ZW AND XX/XY SEX-DETERMINATION SYSTEMS IN BIGHEAD CATFISH (CLARIAS MACROCEPHALUS, GÜNTHER, 1864), NORTH AFRICAN CATFISH (CLARIAS GARIEPINUS, BURCHELL, 1822), AND HYBRID CATFISH (CLARIAS MACROCEPHALUS X CLARIAS GARIEPINUS)

NGUYEN HO MY DUNG

GRADUATE SCHOOL, KASETSART UNIVERSITY
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THESIS

AN INVESTIGATION OF ZZ/ZW AND XX/XY SEX-DETERMINATION SYSTEMS IN BIGHEAD CATFISH (*CLARIAS MACROCEPHALUS*, GÜNTHER, 1864), NORTH AFRICAN CATFISH (*CLARIAS GARIEPINUS*, BURCHELL, 1822), AND HYBRID CATFISH (*CLARIAS MACROCEPHALUS* X *CLARIAS GARIEPINUS*)

NGUYEN HO MY DUNG

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
Doctor of Philosophy (Genetics)
Graduate School, Kasetsart University
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NGUYEN HO MY DUNG : An Investigation of ZZ/ZW and XX/XY Sex-Determination Systems in Bighead Catfish (*Clarias macrocephalus,* Günther, 1864), North African Catfish (*Clarias gariepinus,* Burchell, 1822), and Hybrid Catfish (*Clarias macrocephalus* x *Clarias gariepinus*). Doctor of Philosophy (Genetics), Major Field: Genetics, Department of Genetics.
Thesis Advisor: Associate Professor Kornsorn Srikulnath, Ph.D.
Academic Year 2021

Aquaculture plays an important role in the food and nutrition of many countries and is particularly significant in rural development. Clariid catfish (*Clarias* spp.) are regarded as one of the most promising aquaculture species and contribute significantly to the economy of Thailand. The hybrid freshwater catfish from native parental species (*C. gariepinus* x *C. macrocephalus*) has faster growth and disease resistance as a favored breed in aquaculture but the sterility of F_1 male hybrids has presented a major obstacle to commercial production. The complex sex-determination system (SDS) between the two parental species might affect sterility. This study explored the SDSs of these two species using diversity arrays technology sequencing (DArTseq) to generate single nucleotide polymorphism (SNP) loci and restriction site specific presence-absence (PA) markers. DArTseq is a successful method for identifying sex-linked markers in non-model species. Male and female-linked loci reached the criteria of moderately sex-linked loci at 70:30, 80:20, 90:10 and 100:0. Results suggest that the male heterogametic XX/XY SDS should co-exist with the ZZ/ZW system in African catfish. The SDS of African catfish might be influenced by a polygenic sex-determination (PSD) system, consisting of many independently segregating sex “switch” loci to determine sex within a species, while bighead catfish may also exhibit a male heterogametic XX/XY sex-determination system. One male-linked locus showed homology with the *GTSF1L* gene, which exhibits a testis-enriched expression pattern. Male-linked loci on the putative Y sex chromosome were identified as an extremely small proportion of the genome. A PCR-based DNA marker was developed to validate the male-linked loci in bighead catfish. Research results disclosed that ZZ/ZW SDS can co-exist with XX/XY SDS as PSD in the same individuals of hybrid catfish. Most of these loci were not sex-linked in the parental species, suggesting that the hybrid exhibits a combination of different alleles. One female-linked locus was homologous with the *B4GALNT1* gene, which is involved in the spermatogenesis pathway and hatchability. These SDS findings can be applied to improve commercial breeding of this species in Thailand. This approach, using moderately sex-linked loci, provides a baseline to identify potential sex determination regions in catfish, thereby enabling genetic enhancement in breeding programs.

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Student's signature Thesis Advisor's signature

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NGUYEN HO MY DUNG

TABLE OF CONTENTS

Page

List of abbreviations

SDS	=	Sex-determination system
PSD	=	Polygenic sex-determining mechanisms
GSD	=	Genetic sex-determination systems
ESD	=	Environmental sex-determination systems
RFLP	=	Restriction fragment length polymorphism
AFLP	=	Amplified fragment length polymorphism
RADseq	=	Restriction site-associated DNA sequencing
PA	=	Presence-Absence
SNP	=	Single nucleotide polymorphisms
DArTseq	=	Diversity Arrays Technology

Introduction

Aquaculture plays an important role in the food and nutrition of many countries and is particularly significant in rural development (Loring, 2019). *Clarias* catfish, including North African catfish (*Clarias gariepinus*), bighead catfish (*C. macrocephalus*) and their hybrids are farmed in at least 55 countries with an annual global output of 1, 245.3 million tons in 2018 (FAO, 2021). The bighead catfish (*C. macrocephalus*) is widely distributed in Laos, Cambodia, Vietnam, and Thailand (Davidson, 1975; Kuronuma, 1961; Rainboth, 1996; Suvatii, 1981). The bighead catfish is preferred for its better taste and soft meat but aquaculture is not widespread because of slow growth and high disease sensitivity (Senanan et al., 2004). The North African catfish (*C. gariepinus*) is a highly adaptable species with tolerance for low water quality and resistance to many infectious agents. During the 1980s, a hybrid catfish was introduced by crossing female bighead catfish (*C. macrocephalus*) and male African catfish (*C. gariepinus*). The hybrid has fast growth and high disease resistance contributed by the parental genomes and became popular in aquaculture (Na-Nakorn et al., 1994), accounting for over 90% of catfish production in Thailand in 2004 (Na-Nakorn, Kamonrat, et al., 2004; Ponjarat et al., 2019). However, the sterility of the hybrid is a major obstacle to mass production. The SDS complexity between the two parental species has been postulated to impact sterility (Na-Nakorn, Rangsin, et al., 2004; Ponjarat et al., 2019) SDSs are complex and poorly understood in catfish. Breeding programs encounter difficulties in sex manipulation to produce monosex populations that can support the stability of mating systems.

Background and Rationale

Sex determination systems in teleosts have been observed across taxa as hermaphroditism, genetic sex determination (GSD), multifactorial polygenic sex-determining (PSD) mechanisms and environmental sex determination (ESD) (Ezaz et al., 2016). The evolutionary lability of sex determination and the rapid rate of turnovers and transitions among different modes makes the teleost an excellent model for testing theories regarding the evolution of sex determining adaptation (Pennell et al., 2018). Heterogametic sex chromosomes have been observed in less than 1% of teleost fish (Kottler & Schartl, 2018; Mank et al., 2006), while sex chromosomes in teleosts are far less differentiated, with recombination often occurring along their length. In the catfish lineage, sex determination processes are complex, with varying patterns of sex chromosomes depending on geographical region. Clariid catfish include 14 genera found in India, Syria, Southeast Asia and Africa. Most clariid species have the diploid chromosome number (2n) ranging from 48 to 56, except for *C. pachynema* (2n = 66) and one population of *C. batrachus* (2n = 104) (Eyo & Effiong, 2005; Jianxun et al., 1991; Malla & Ganesh, 2009; Maneechot et al., 2016). In the *Clarias* genus, walking catfish, mudfish (*C. anguillaris*, Linnaeus, 1758) and West African catfish (*C. ebriensis*, Pellegrin, 1920) have a ZZ/ZW sex-determination system (Eyo & Effiong, 2005; Pandey & Lakra, 1997), whereas white spotted *clarias* show XX/XY.

The African catfish is the most complex species with XX/XY or ZZ/ZW systems in different geographical populations. Almost all the reported sex chromosome systems from Africa and Israel show that *C. gariepinus* indicated a ZZ/ZW system (Eyo & Effiong, 2005; Ozouf-Costaz et al., 1990; Teugels et al., 1992). Molecular marker, chromosome analysis, genetic manipulation and hormonal sex-reversal experiments suggested a XX/XY system in *C. gariepinus* with different sampled populations from Israel, Hungary and China (Eding, 1997; Galbusera et al., 2000; Liu et al., 1996). In bighead catfish, no heteromorphic sex chromosomes have been identified (Maneechot et al., 2016; Ponjarat et al., 2019; Siraj et al., 2009), while gynogenesis research can induce bighead catfish to produce all-female stocks. This study suggests a possible male heterogametic (XX/XY) system (Na-Nakorn, 1995).

Previous studies identified diploid chromosome numbers of African catfish and bighead catfish as 56 and 54, respectively while the diploid chromosome number of hybrid catfish is 55 (Maneechot et al., 2016; Ponjarat et al., 2019). The male hybrid produced as a result of interspecific hybridization is sterile, and widespread production of the F_1 hybrid has been limited. This sterility might occur because of the different parental diploid chromosome numbers or from a complex interaction between the two different sex-determining mechanisms of these species (Na-Nakorn, Rangsin, et al., 2004; Ponjarat et al., 2019). Therefore, understanding the sex-determination system of *C. gariepinus*, *C. macrocephalus* and hybrid catfish in Thailand is necessary to assist in breeding programs of this species and its hybrid.

Various methods are used to identify sex chromosomes such as karyotype analyses prepared using standardized staining procedures that reveal characteristic structural features for heteromorphic sex chromosomes in teleosts and other animals (Chen et al., 2014; Singchat et al., 2020; Singchat et al., 2018; Srikulnath et al., 2019; Srikulnath et al., 2014; Srikulnath et al., 2015). However, this approach is not available for several species that exhibit homomorphic sex chromosomes (Panthum et al., 2021; Ponjarat et al., 2019; Singchat et al., 2020). Molecular genetic markers, restriction fragment length polymorphism (RFLP) and amplified fragment length polymorphism (AFLP) were applied in several studies (Bağda et al., 2013; Kovács et al., 2000; Vaux et al., 2020) but these traditional methods are time-consuming. In taxa with homomorphic sex chromosomes, restriction site-associated DNA sequencing (RADseq) has been proposed as an approach to identify sex-linked marker (Gamble & Zarkower, 2014). This method has proved successful in inferring the sex determining process for several fish species (Palaiokostas et al., 2013). Currently, diversity arrays technology (DArT) based on next-generation sequencing technology is a whole-genome genotyping tool that can discover and score hundreds of polymorphic loci without the requirement for preexisting sequence information. DArT is a potent high-throughput approach at cheap cost per data point for obtaining accurate and reproducible marker data. DArT is similar to RAD sequencing, ddRAD sequencing and genotyping by sequencing (GBS) methods to generate sex-linked markers and determine sex-linked regions with multiple loci. Here, a reliable molecular approach based on sex-linked markers was developed to identify the genetic sex of individuals.

Results can provide information about sex chromosome evolution and conservation in several species (Alam et al., 2021; Lambert et al., 2016). Lambert et al. (2016) used single nucleotide polymorphic (SNP) loci generating restriction site specific presence-absence (PA) markers as an effective method to identify sex-linked markers in non-model species. Markers generated using DArTseq™ showed loci assorted to a particular sex. This method enables the identification of loci tightly linked to sex-determining regions of sex chromosomes, thereby providing a useful tool to discover sex-determining modes in non-model species with cryptic sex chromosomes. These sex-linked markers can identify sex reversal in non-model species by observing the discordance between genotypic and phenotypic sex (Lambert et al., 2016).

Objectives

1. To identify the sex determination systems in North African catfish (*C. gariepinus*) and bighead catfish (*C. macrocephalus*).
2. To understand the mechanism of sex determination transfer from parental species to hybrid offspring.

Contribution/Outcome of this Research

Successful development of this research will increase the understanding of sex determination mechanisms, leading to the application of genotypic sex assay and genetic improvement in the breeding management of clariid catfish. All sex-linked loci are necessary to improve sex control in monosex populations, protect wild populations through supportive breeding and maintain effective population size. The research findings will assist future studies, provide useful data for understanding the genetic basis of sex determination and discover novel genes and sequences in sex differentiation pathways.

Publications

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An Investigation of ZZ/ZW and XX/XY Sex Determination Systems in North African Catfish (*Clarias gariepinus*, Burchell, 1822)

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An investigation of sex-specific loci may provide important insights into fish sex determination strategies. This may be useful for biotechnological purposes, for example, to produce all-male or all-female fish for commercial breeding. The North African catfish species, *Clarias gariepinus*, has been widely adopted for aquaculture because its superior growth and disease resistance render the species suitable for hybridization with other catfish to improve the productivity and quality of fish meat. This species has either a ZZ/ZW or XX/XY sex determination system. Here, we investigate and characterize these systems using high-throughput genome complexity reduction sequencing as Diversity Arrays Technology. This approach was effective in identifying moderately sex-linked loci with both single-nucleotide polymorphisms (SNPs) and restriction fragment presence/absence (PA) markers in 30 perfectly sexed individuals of *C. gariepinus*. However, SNPs based markers were not found in this study. In total, 41 loci met the criteria for being moderately male-linked (with male vs. female ratios 80:20 and 70:30), while 25 loci were found to be moderately linked to female sex. No strictly male- or female-linked loci were detected. Seven moderately male-linked loci were partially homologous to some classes of transposable elements and three moderately male-linked loci were partially homologous to functional genes. Our data showed that the male heterogametic XX/XY sex determination system should co-exist with the ZZ/ZW system in *C. gariepinus*. Our finding of the co-existence of XX/XY and ZZ/ZW systems can be applied to benefit commercial breeding of this species in

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Thailand. This approach using moderately sex-linked loci provides a solid baseline for revealing sex determination mechanisms and identify potential sex determination regions in catfish, allowing further investigation of genetic improvements in breeding programs.

Keywords: fish, SNP, recombination, aquaculture, transposable element

INTRODUCTION

Aquaculture plays an important role in global food production (Belton and Thilsted, 2014; Loring et al., 2019). However, the availability of land and water resources for aquaculture is currently limited, while susceptibility to new diseases is increasing (Ayyam et al., 2019; Lebel et al., 2019). This has facilitated continuous improvements in breeding programs by cross-breeding between different species to generate new hybrid varieties that can tolerate high stocking densities (Dunham and Masser, 2012; Zhou et al., 2018). Clariid catfish (*Clarias* spp.) are commonly distributed in freshwater from the River Orange in South Africa to the Nile in North Africa, as well as in parts of Asia (Israel, Syria, Southern Turkey, and Southeast Asia) (Skelton and Teugels, 1992; Teugels and Adriaens, 2003; Na-Nakorn et al., 2004a). With good flesh quality (taste and firmness), the bighead catfish (*Clarias macrocephalus*, Günther, 1864) is one of the most economically important freshwater fish in Southeast Asia (Günther, 1864; Teugels et al., 1999). However, it is difficult to develop sustainable breeding management programs because of the low growth rate and high susceptibility to disease (Jarimopas et al., 1988; Na-Nakorn et al., 1993; Senanan et al., 2004). The North African catfish species (*C. gariepinus*, Burchell, 1822) was introduced for hybridization with bighead catfish to improve the productivity and quality of the fish meat (Burchell, 1822; Nukwan et al., 1990). A hybrid was developed by crossing artificially male North African catfish and female bighead catfish (Senanan et al., 2004). This F_1 hybrid catfish exhibits a rapid growth rate and has a high disease resistance, which has led to the propagation of these hybrids in the aquaculture market. They now represent more than 90% of catfish production in Thailand (Na-Nakorn et al., 2004a). The F_1 hybrids are physically more vigorous than either parental species, but their mass production has been limited by reproductive failure (Koolboon et al., 2014; Ponjarat et al., 2019). Ponjarat et al. (2019) asserted that hybrid dysgenesis between the bighead catfish and North African catfish is caused by karyotypic and genomic differences, resulting in meiotic arrest and subsequent apoptosis of gametocytes. However, the F_1 hybrid can occasionally cross-breed in captivity, whereby the female hybrids are fertile, with the potential to produce large numbers of backcross progeny that shows fertility and low embryo mortality, whereas the male hybrids are sterile (Na-Nakorn et al., 2004b; Abol-Munafi et al., 2006). Sex hormones have been applied in an attempt to promote spermatogenesis, but the F_1 male hybrids have always remained sterile (Abol-Munafi et al., 2006). The complexity of sex determination interaction between the two species might influence differences in fertility between F_1 male and female hybrids (Ponjarat et al., 2019).

The North African catfish species, *C. gariepinus* has been widely adopted for aquaculture within and outside its native ranges (Van der Bank, 1998; Dai et al., 2011; Wachirachaikarn and Na-Nakorn, 2019), and is considered the tropical catfish species best suited to aquaculture (Clay, 1979; Okonkwo and Obiakor, 2010), leading to the application of *C. gariepinus* for cross-breeding with other clariid catfish (Rahman et al., 2013). They have been extensively distributed around the world (Vitule et al., 2006; Na-Nakorn and Brummentt, 2009). The farming of *C. gariepinus* is rapidly expanding due to its superior growth and disease resistance against fungi, viruses and bacteria (Akoll and Mwanja, 2012; Taukhid et al., 2015). *C. gariepinus* that carried the MHC-II marker showed better growth and were significantly different from other catfish (Suprapto et al., 2017). Moreover, they are also important for biological research such as genome manipulation (Bongers et al., 1995; Galbusera et al., 2000; Erondu et al., 2011; Nwachi and Dasuki, 2017). However, the problematics of taxonomy and systematics are complex and need thorough revision with inclusion of populations from different geographic origins (Teugels, 1982, 1984, 1986). Historically, several stocks of *C. gariepinus* have been introduced to Thailand (Wachirachaikarn and Na-Nakorn, 2019), and genetic diversity of a few has been identified significantly different between Thailand and Nigeria (Falaye et al., 2011; Wachirachaikarn and Na-Nakorn, 2019). Therefore, a proper understanding of the sex determination system of *C. gariepinus* in Thailand is necessary to assist in breeding programs of this species and its hybrid.

The family of air-breathing or labyrinth catfish (Clariidae) is one of the largest among the Siluriformes (Burgess, 1989). Clariid fish comprise 14 genera with 115 species found in India, Syria, Southern Turkey, Southeast Asia, and Africa. Most have the highly conserved karyotype with diploid chromosome number (2n) ranging from 48 to 56, with the exception of *C. pachynema* (2n = 66), and one population of *C. batrachus* (2n = 104) (Jianxun et al., 1991; Eyo and Effiong, 2005; Malla and Ganesh, 2009; Maneechot et al., 2016). These findings show that *C. gariepinus* phylogenetically exhibits both the XX/XY and ZZ/ZW system (**Figure 1**). However, significant variation in fish sex chromosome systems has been recorded, not only among closely related species (such as in tilapias, ricefishes, or sticklebacks; Takehana et al., 2008; Ross et al., 2009; Ser et al., 2010; Cnaani, 2013) but also between different populations of the same species (e.g., *Eigenmannia virescens* and *Ancistrus* cf. *dubius*; Almeida-Toledo et al., 2001; Mariotto et al., 2004; Mariotto and Miyazawa, 2006; Fernandes et al., 2019). Recent genotyping using next-generation sequencing, such as Diversity Arrays Technology (DArTseq™) developed by Diversity Arrays Technology Pty Ltd., (Canberra, ACT, Australia), is an effective method for the identification of sex-linked loci in non-model species. The variability in SNP

loci generates presence/absence polymorphism in restriction sites (so-called PA markers) and these may facilitate the identification of divergent genomic regions present in one sex only, thus pointing to a putative region of suppressed recombination on a presumed sex chromosome. To identify the sex determination system in the *C. gariepinus*, we applied DArTseq™ in captive-bred individuals scored with phenotypic sex. The mapped DArTseq™ sequences were then used to search for homologies with other 'model' teleosts (Japanese rice fish: *Oryzias latipes*, zebrafish: *Danio rerio*, and Japanese pufferfish: *Takifugu rubripes*), and amniotes (chicken: *Gallus gallus*). Our findings also provide novel insights into the evolutionary history of sex determination in catfish.

MATERIALS AND METHODS

Specimens and DNA Extraction

Fifteen male and fifteen female individuals of North African catfish (*C. gariepinus*) were collected with weight of 1.7–2.0 kg and length of 35–40 cm. The sampled individuals were randomly picked from a large breeding stock to avoid the probability of high incidence of siblings in our pool. The sex of each individual was identified by external morphology and internal examination of gonadal morphology (Kitano et al., 2007; Esmaeili et al., 2017; Ponjarat et al., 2019). Hence, the sex of all specimens obtained from the Department of Aquaculture, Faculty of Fisheries, Kasetsart University (Bangkok, Thailand) was determined to avoid potentially wrong sexing due to sex reversal. The fins were removed for DNA extraction. Animal care and all experimental procedures were approved by the Animal Experiment Committee, Kasetsart University (Approval No. ACKU61-SCI-026), and conducted in accordance with the Regulations on Animal Experiments at Kasetsart University. Whole genomic DNA was extracted following the standard salting-out protocol as previously described (Supikamolseni et al., 2015). The quality of extracted DNA was evaluated by gel electrophoresis, and samples with high-molecular weight DNA were stored at −20°C until required for DArTseq™ library construction.

DArT Sequencing and Genotyping

A detailed description of the DArTseq™ methodology can be found in Jaccoud et al. (2001). The method often produces 69 base pairs (bp) long sequences. Genotyping of multiple loci was performed using DArTseq™ (Diversity Arrays Technology Pty Ltd., Canberra, Australian Capital Territory, Australia) for SNP loci and *in silico* DArT (PA of restriction fragments in the representation; PA loci) to determine the candidate sex-specific loci between male and female individuals. Approximately 100 ng of DNA from each sample was used for the development of DArTseq™ arrays. DNA samples were subjected to digestion/ligation reactions as described by Kilian et al. (2012) and digested with *Pst*I and a second restriction endonuclease (*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 a *Sph*I compatible adaptor consisting of an Illumina flow-cell attachment region. Ligated fragments were then amplified by PCR using the following parameters: 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[1] bridge PCR followed by sequencing on the Illumina HiSeq 2000 platform. Single read sequencing was run for 77 cycles.

Sequences were processed using proprietary DArTseq™ analytical pipelines (Ren et al., 2015). Initially, the HiSeq 2000 output (FASTQ file) was processed to filter poor-quality sequences. Two different thresholds of quality were applied. For the barcode region (allowing parsing of sequences into specific sample libraries), we applied stringent selection (minimum Phred pass score of 30, minimum pass length percentage 75). For the remainder of the sequence, relaxed thresholds were applied (minimum Phred pass score 10, minimum pass length percentage 50). Approximately 2,000,000 sequences per individual were identified and used in marker calling. Finally, identical sequences were combined into "fastqcoll" files that were used in the secondary proprietary pipeline (DArTsoft14) for SNP and PA loci calling. To this end, we used the "reference-free" algorithm implemented in DArTsoft14. The sequence clusters were then parsed into SNP and *in silico* DArTseq™ markers utilizing a range of metadata parameters derived from the quantity and distribution of each sequence across all samples in the analysis. Multiple libraries of the same individual were included in the DArTseq™ genotyping process, enabling reproducibility scores to be calculated for each candidate marker. Outputs by DArTsoft14 were then filtered on the basis of reproducibility values, average count for each sequence (sequencing depth), balance of average counts for each SNP allele, and call-rate (proportion of samples for which the marker was scored).

Marker Selection and DArT Sequencing Analysis

Sex-specific loci were derived from the analysis of SNP co-dominant markers and PA as dominant markers. The SNP data were coded as "0" for the reference allele homozygote (the most common allele), "1" for the SNP allele homozygote, "2" for the heterozygote, or "–" as the double null/null allele homozygote (absence of a SNP fragment in the genomic representation). The PA data were coded as "1" for presence, "0" for absence, or "–" for putative heterozygosity. For sex-linked markers in an XX/XY sex-determination system, reference alleles are expected to be located on the X-chromosome. Here, "SNP alleles" were those that showed polymorphism relative to the reference allele. In an XX/XY system, SNP alleles should be associated with the Y sex chromosome, and located in or near to the male-determination region if the allele is tightly Y-specific. If the two sex chromosomes recombine, SNP alleles should occasionally appear on the X chromosome. Some males might then be homozygous for SNP alleles at particular loci, with females

[1]http://www.illumina.com/products/cbot.html

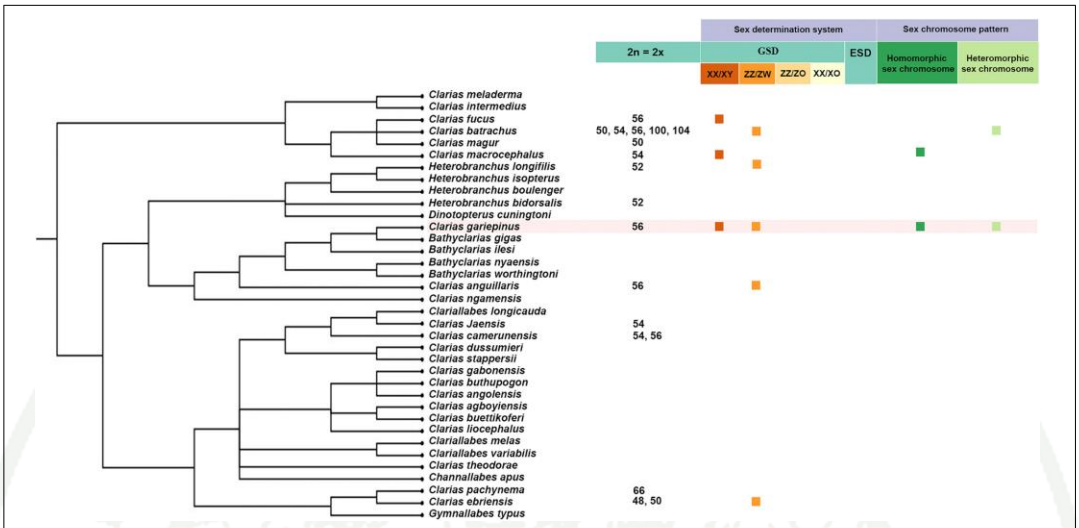

FIGURE 1 | Phylogenetic tree showing number of chromosomes and sex determination system of the Clariidae family (Ozouf-Costaz et al., 1990; Teugels et al., 1992; Na-Nakorn, 1995; Bakhoum, 1996; Liu et al., 1996; Eding et al., 1997; Pandey and Lakra, 1997; Kovács et al., 2001; Eyo and Effiong, 2005; Malla and Ganesh, 2009; Okonkwo and Obiakor, 2010; Awodiran et al., 2015; Maneechot et al., 2016).

being heterozygous, exhibiting a copy of the SNP allele but the probability of a female being homozygous for a SNP allele should be low. For evaluation of loci associated with an XX/XY system, loci with female homozygosity frequencies for the reference allele of at least 70% were retained, whereas those with homozygosity at the SNP allele at no more than 30% and heterozygosity at no more than 30% were discarded. For males, loci with homozygosity frequencies for the reference allele of at most 30% and heterozygosity of at least 70% were retained. However, this allowed sex-linked loci to show a higher degree of SNP allele homozygosity for males if recombination occurred. For PA markers, loci that had restriction fragments sequenced in at least 70% of males and not sequenced in at least 70% of females were selected. The SNP and PA loci sequenced for 80%, 90%, and 100% of males were also included in a separate dataset. Loci passing the 100% filtering criterion were designated as perfectly sex-linked, whereas those passing at 70–90% were called moderately sex-linked loci. An opposite similar approach was carried out for targeting loci with a ZZ/ZW system.

Calculation of the Hamming distance was performed to determine the number of combined loci between male and female individuals for pairwise differences in SNP and PA loci using the "rdist" function of R version 3.5.1 statistical software (R Core Team, 2019). Heatmaps were plotted using the ggplot2 R package (R Core Team, 2019). The Hamming distance represents the number of pairwise differences between all individuals across all loci. To examine the genetic association between each locus and phenotypic sex assignment from the SNP and PA loci, the Cochran-Armitage trend test (CATT) was performed using the "catt" function of R version 3.5.1 with the HapEstXXR package (R Core Team, 2019; Sven and Klaus, 2019). The CATT results were similar to those of a chi-square test to assess whether the proportion of different genotypes followed the null expectation. Polymorphism information content (PIC), as an index for evaluation of the informativeness of SNP and PA loci, was calculated for each locus and ranged from 0 (fixation of one allele) to 0.5 (frequencies of the two alleles are equal).

Estimation of Expected Sex-Linked Markers

The probability of candidate loci showing random associations with sex under a small sample size was estimated (Lambert et al., 2016). The formula $P_i = 0.5^n$ describes the probability of a locus being perfectly sex-linked by chance, 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. After sequencing, we multiplied P by the number of high-quality SNPs produced to estimate the number of SNPs expected to exhibit a perfectly sex-linked pattern by chance.

Comparison of Potential Sex-Linked Loci

We scored all candidate loci, the frequency of analyzed loci was not strictly (100%) sex-linked. A three-sample chi-square for PA loci and heterozygosity for SNP loci assuming unequal variances (based on results from the descriptive statistics) was performed to determine whether these three groups were significantly different from each other using the chi-square test with the R package "stats" for PA loci, and the Kruskal-Wallis test using the R package "stats" and the Nemenyi test using the R package

"PMCMR" for SNP loci, to determine the mean heterozygosity and standard deviation for each (Gruber and Georges, 2019). All candidate loci were plotted against each individual using the 'glPlot' function in the R package 'dartR' (Gruber and Georges, 2019; R Core Team, 2019).

Homology Searching

For all sex-linked loci that reached our criteria and had statistically significant associations with phenotypic sex, a Basic Local Alignment Search Tool (BLAST) search was performed to investigate the homologies of the sex-linked SNP/PA loci against a selection of available teleost fish genomes (Japanese rice fish: *O. latipes*) (Accession No. GCF_002234675.1) (Kasahara et al., 2007), zebrafish: *D. rerio* (Accession No. GCA_000002035.4) (Broughton et al., 2001), Japanese pufferfish: *T. rubripes* (Accession No. GCA_901000725.2) (Elmerot et al., 2002), amniote reference genomes (chicken: *G. gallus*) (Accession No. AADN00000000.5) (International Chicken Genome Sequencing Consortium, 2004). We selected the aforementioned species as representative reference genomes in the homology search analysis due to the availability of their high-quality gene annotations and near complete up-to-date assemblies. The BLAST homology search was performed in two rounds. First we aligned sex-linked loci against the reference fish genome, and then we mapped the homologous genes to further clarify their location on sex chromosomes of high-quality annotated genomes representing vertebrate and invertebrate species. Using the BLASTn program, sex-linked loci were used to search the NCBI database[2] and RepBase version 19.11 (Bao et al., 2015) (Genetic Information Research Institute, http://www.girinst.org/). This is a specialized nucleotide sequence collection for repeated or other significant sequences that only reports E-values lower than 0.05 and query coverage with similarity of more than 50%.

RESULTS

Determination of Sex-Linked Loci in North African Catfish

We sequenced 42,752 SNP loci and 118,118 PA loci. PIC values ranged from 0.49 to 0.50 for all loci. To determine whether XX/XY or ZZ/ZW sex chromosomes drive sex determination in *C. gariepinus*, we compared a number of SNP and PA after filtering with a gradually varying set of criteria. For the ZZ/ZW type, filtering using the criterion of 30:70 male:female presented only 25 PA loci as moderately female-linked and no SNP loci. Proportional pairwise Hamming distance between males and females using moderately sex-linked PA loci showed within-sex distances of 0.563 ± 0.011 in males and 0.403 ± 0.014 in females, and showed between-sex distances of 0.675 ± 0.007 for PA loci. The CATT results verified a significant locus association with phenotypic sex for 25 PA ($\chi 2$ = 5.07–11.63; $p < 0.001$) loci. Moreover, 20:80 male:female yielded only one PA loci as moderately female-linked and no SNP loci. Hamming distance between males and females using moderately sex-linked PA loci showed within-sex distances of 0.543 ± 0.049 in males and 0.343 ± 0.047 in females. Between-sex distances showed 0.707 ± 0.03 PA loci. CATT verified a significant locus association with sex phenotype for one PA ($\chi 2$ = 9.07; $p < 0.001$) locus (**Figure 2**). No SNP or PA loci associated with females was found for the criteria of 10:90 and 0:100 male:female (**Figure 3**, **Table 1**). Chi-square tests showed that 30:70 and 20:80 filtering criteria indicated no significant differences in males ($\chi 2 = 1.2226 \times 10^{-31}$; $p = 1$) and females ($\chi 2 = 1.0206 \times 10^{-31}$; $p = 1$) for PA.

By contrast, for the XX/XY type, filtering using the criterion of 70:30 male:female yielded 42 PA loci as moderately male-linked loci and no SNP loci. Proportional pairwise Hamming distance between male and female African catfish using moderately sex-linked PA loci (under the null exclusive model) showed lower within-sex distances of 0.373 ± 0.01 in males and 0.624 ± 0.009 in females for PA loci. Between-sex distances showed 0.699 ± 0.006 for PA loci. CATT verified significant loci association with phenotypic sex for 41 PA ($\chi 2$ = 3.55–13.06; $p < 0.001$) loci. The criterion of 80:20 male:female yielded one PA locus as moderately male-linked. Hamming distance between males and females using moderately sex-linked PA loci showed within-sex distances of 0.343 ± 0.047 in males and 0.648 ± 0.047 in females, and between-sex distances of 0.773 ± 0.0028 for PAloci. CATT verified significant loci association with phenotypic sex for one PA ($\chi 2$ = 9.75; $p < 0.001$) locus (**Figure 2**). The criteria of 90:10 and 100:0 male:female did not show any PA locus as male-specific (**Figure 3**, **Table 2**). Chi-square tests revealed that the 70:30 and 80:20 filtering criteria produced no significant differences in males ($\chi 2 = 4.5003 \times 10^{-32}$; $p = 1$) and females ($\chi 2 = 2.4989 \times 10^{-32}$; $p = 1$) for PA. A glPlot revealed that the sample group showed greater similarity between sexes when moderately sex-linked loci were considered in both XX/XY and ZZ/ZW sex determination systems (**Figure 4**).

Estimation of Expected Sex-Linked Markers

For a range of sample sizes and loci, a sample of 30 phenotypically sexed individuals is essential to minimize the probability of selecting less than one spurious sex-linked marker. The P_i probability that a single locus exhibited a perfectly sex-linked pattern by chance is 9.31×10^{-10}. Considering that we analyzed the full dataset (no filtering) of 160,870 (including SNP and PA loci), this translates to an expected number of perfect sex-linked loci due to chance alone of 1.5×10^{-4}.

Homology of Putative Sex-Linked Loci

In terms of a ZZ/ZW sex chromosome system, female moderately sex-linked loci of *C. gariepinus* had sequence homology with Japanese rice fish (*O. latipes*), Japanese pufferfish (*T. rubripes*), and zebrafish (*D. rerio*) genomes on the basis of global BLAST analyses of NCBI databases. No sequence homology was found with the chicken genome (**Supplementary Table 1**). We found that 2 of 25 PA loci were homologous with putative genes: *PCDH2AB3* (E-values 2×10^{-11}, Query Cover 100% and

[2]http://blast.ncbi.nlm.nih.gov/Blast.cgi

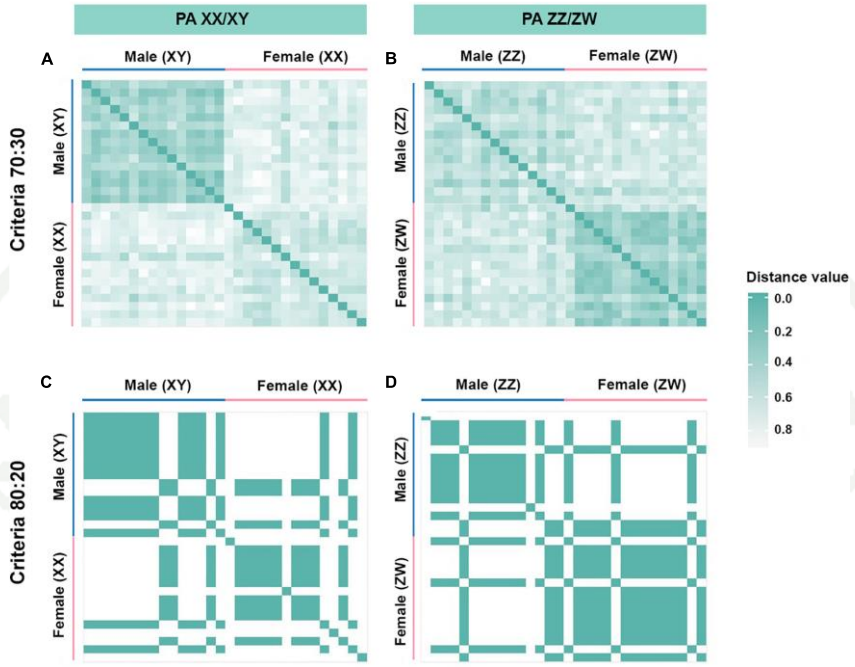

FIGURE 2 | Hamming distance between male and female individuals of African catfish (*Clarias gariepinus*). **(A)** restriction fragment presence/absence (PA) loci with the criterion 70:30 (male:female), **(B)** PA loci with the criterion 30:70 (male:female), **(C)** PA loci with the criterion 80:20 (male:female), **(D)** PA loci with the criterion 20:80 (male:female).

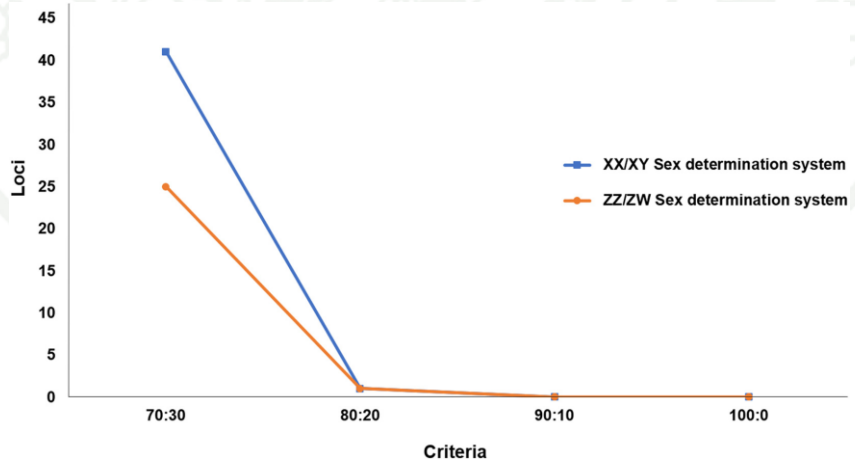

FIGURE 3 | Graph showing number of loci for different hypotheses of sex determination systems after filtering with different criteria. X-axis indicates the number of loci and Y-axis shows the filter criteria.

TABLE 1 | DArT analysis of 15 males and 15 females of African catfish (Clarias gariepinus) (ZZ/ZW sex-determination type).

	30:70 male:female		20:80 male:female		10:90 male:female		0:100 male:female	
	PA[1]	SNP[2]	PA[1]	SNP[2]	PA[1]	SNP[2]	PA[1]	SNP[2]
Total number of DArT analyses	118,118	42,752	118,118	42,752	118,118	42,752	118,118	42,752
Moderately sex-linked loci	25	-	1	-	-	-	-	-
Overall mean distance between males and females	0.675 ± 0.007	-	0.707 ± 0.03	-	-	-	-	-
Overall mean distance within females	0.403 ± 0.014	-	0.343 ± 0.047	-	-	-	-	-
Overall mean distance within males	0.563 ± 0.011	-	0.543 ± 0.049	-	-	-	-	-

[1]*Presence/Absence (PA) loci.*
[2]*Single nucleotide polymorphism (SNP) loci.*

TABLE 2 | DArT analysis of 15 males and 15 females of African catfish (*Clarias gariepinus*) (XX/XY sex-determination type).

	70:30 male:female		80:20 male:female		90:10 male:female		100:0 male:female	
	PA[1]	SNP[2]	PA[1]	SNP[2]	PA[1]	SNP[2]	PA[1]	SNP[2]
Total number of DArT analyses	118,118	42,752	118,118	42,752	118,118	42,752	118,118	42,752
Moderately sex-linked loci	41	-	1	-	-	-	-	-
Overall mean distance between males and females	0.699 ± 0.006	-	0.773 ± 0.0028	-	-	-	-	-
Overall mean distance within females	0.624 ± 0.009	-	0.648 ± 0.047	-	-	-	-	-
Overall mean distance within males	0.373 ± 0.01	-	0.343 ± 0.047	-	-	-	-	-

[1]*Presence/Absence (PA) loci.*
[2]*Single nucleotide polymorphism (SNP) loci.*

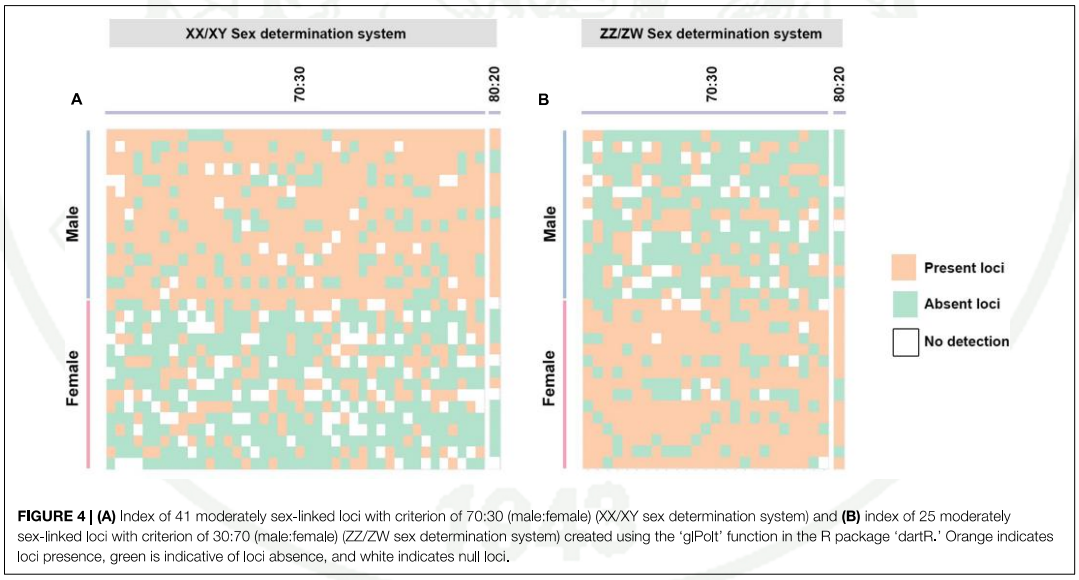

FIGURE 4 | (A) Index of 41 moderately sex-linked loci with criterion of 70:30 (male:female) (XX/XY sex determination system) and **(B)** index of 25 moderately sex-linked loci with criterion of 30:70 (male:female) (ZZ/ZW sex determination system) created using the 'glPolt' function in the R package 'dartR.' Orange indicates loci presence, green is indicative of loci absence, and white indicates null loci.

similarity 82.61%) and *DCTN4* (E-values 4×10^{-8}, Query Cover 68% and similarity 89.58%) from *D. rerio* (Wu, 2005; Bayés et al., 2017) (**Supplementary Table 2**). Moreover, 4 of 25 PA loci had partial homology with transposable elements, comprising 3 loci with similarity to *Gypsy* families and 1 locus of retroelement *Rex1* (**Supplementary Table 3**). By contrast, for the XX/XY sex chromosome system, 3 out of 41 moderately male sex-linked PA loci of *C. gariepinus* had sequence homology with putative genes: *ADD3* (E-values 2×10^{-4}, Query Cover 71% and similarity 81.63%) from *D. rerio* (Pasquier et al., 2016), *GUCD1* (E-values 0.038, Query Cover 56% and similarity 82.05%) from *T. rubripes* (Elmerot et al., 2002), and *DNTA* (E-values 0.003,

Query Cover 52% and similarity 86.11%) from *G. gallus* (Bellott et al., 2017) (**Supplementary Table 4**), whereas 7 of 41 PA loci had partial homology with transposable elements consisting of 3 loci of *Gypsy*, 3 loci of *Tc1/mariner* families, and 1 locus of short interspersed nuclear elements (*SINEs*) (**Supplementary Table 5**). Moreover, one PA locus was matched with a non-coding region homologous to the chicken Z chromosome (GGAZ) (**Supplementary Table 6**).

DISCUSSION

Fish sex chromosomes are often homomorphic (i.e., cytologically indistinguishable) which hampers their analysis (Otake et al., 2010; Gammerdinger and Kocher, 2018). Such a condition, however, seems to facilitate frequent sex chromosome turnovers (Gammerdinger and Kocher, 2018; Ieda et al., 2018). The variation in fish sex chromosome systems has been recorded among closely related species (such as in tilapias, ricefishes, or sticklebacks; Takehana et al., 2008; Ross et al., 2009; Ser et al., 2010; Cnaani, 2013) and also between different populations of the same species (e.g., *Eigenmannia virescens* and *Ancistrus* cf. *dubius*; Almeida-Toledo et al., 2001; Mariotto et al., 2004; Mariotto and Miyazawa, 2006; Fernandes et al., 2019). Given these facts, the identification of sex-linked loci is important for a thorough elucidation of the sex chromosome origin and evolution in teleosts, on both inter-specific and inter-population levels (Herpin and Schartl, 2015; Kottler and Schartl, 2018). An intriguing situation has been found in *C. gariepinus*, where several reports differed in the type of detected sex chromosome systems, suggesting either XX/XY or ZZ/ZW constitution (Ozouf-Costaz et al., 1990; Bakhoum, 1996; Liu et al., 1996; Eding et al., 1997; Váradi et al., 1999; Okonkwo and Obiakor, 2010). Here, DArTseq^{TM} technology was applied to a large number of SNP/PA loci, allowing the prediction of sex-linked loci for *C. gariepinus* using a sample size of 30 individuals (15 males and 15 females). Many false-positive signals might be expected from such specimens due to their more diverse genetic background (Gamble et al., 2015); however, our data show the feasibility of our approach. The probability of one locus showing sex linkage spuriously was 9.31×10^{-10} from the full dataset (no filtering) of 160,870 loci (including SNP and PA loci), whereas the expected amount of perfect sex linkage was estimated to be 1.5×10^{-4}; thus, identification of any sex-linked loci by chance seems unlikely. Our analysis reports the co-occurrence of male and female linked loci with a different proportion (70:30) across all the sampled specimens, suggesting that the XY and ZW systems can coexist inside the same fish individual. Our results indicate that sex chromosome turnover might still be in action in this species.

Both ZZ/ZW or XX/XY Sex Chromosome Systems Co-exist in North African Catfish

Most of the reported sex chromosome systems from Africa and Israel support the assumption that the ZZ/ZW system is the ancestral type for *C. gariepinus* (Ozouf-Costaz et al., 1990; Teugels et al., 1992; Eyo and Effiong, 2005). However, molecular marker, chromosome analysis, genome manipulation (both gynogenesis and androgenesis), and hormonal sex-reversal experiments with different sampled populations from Israel, Hungary and China have suggested a XX/XY system in *C. gariepinus* (Liu et al., 1996; Eding et al., 1997; Galbusera et al., 2000; **Figure 5**). Our study successfully identifies 41 SNP/PA loci as moderately male-linked loci, in contrast to 25 moderately female-linked loci. Based on the comparison between several teleost reference genomes, the loci were not all located in the same linkage group. One scenario might be that all loci are in the same linkage group for *C. gariepinus* but, just hypothetically, this chromosome pair was evolved by fusion. Comparison of moderately sex-linked loci between the two candidate systems showed that two female-linked loci had homology with putative protein-coding genes. One of these two loci was homologous to the *DCTN4* gene located on the sex chromosome in platypus (*Ornithorhynchus anatinus*) (Warren et al., 2008) (**Supplementary Table 7**). By contrast, three moderately male-linked loci were homologous to protein-coding genes, including *GUCD1*, which is located on the sex chromosome of the monarch butterfly (*Danaus plexippus plexippus*), tongue sole (*Cynoglossus semilaevis*), fruit fly (*Drosophila busckii*), and dog (*Canis lupus familiaris*) (**Supplementary Table 8**) (Lindblad-Toh et al., 2005; Chen et al., 2014; Gu et al., 2019; Renschler et al., 2019). However, in this study, moderately male-linked markers were found simultaneously with moderately female-linked markers in the same individuals, as found in cichlids (Roberts et al., 2016). This suggests that the XX/XY sex determination system might co-exist with ZZ/ZW system in the same individuals of *C. gariepinus*.

Genomic convergence has been detected by comparative cytogenetics studies in which unrelated sex chromosomes share sex chromosomal linkage homologies across distantly related species (Srikulnath et al., 2014, 2015; Ahmad et al., 2020; Koomgun et al., 2020; Laopichienpong et al., 2020; Singchat et al., 2020a,b). We found that a male-linked locus under the 70:30 criterion shared partial homology with *ADD3* which is localized on GGA6, which is further orthologous to sex chromosomes of sand lizard (*Lacerta agilis*). In addition, another male-linked 70:30 locus had a partial homology with *GUCD1* on GGA15 that is partially homologous to sex chromosomes of green anole (*Anolis carolinensis*), spiny softshell turtle (*Apalone spinifera*), and Chinese softshell turtle (*Pelodiscus sinensis*) (**Figure 6**; Kawagoshi et al., 2009; Alföldi et al., 2011; Badenhorst et al., 2013; Rovatsos et al., 2014; Srikulnath et al., 2014; Singchat et al., 2020a,b). Similar cases were also observed in which linkage homologies or small partial linkage homologies were conserved between teleost and amniote sex chromosomes (Kasahara et al., 2007; Cortez et al., 2014). Our results are only based on observations of certain PA loci showing homology to segments presented on some sex chromosomes of other amniotes or members of gene families, not exactly the identical gene. Moreover, lots of material between sex chromosomes and autosomes moves via transposition (in both directions); therefore, some genes can be independently co-opted to relocate to different linkage groups, with abundant evidence, typically

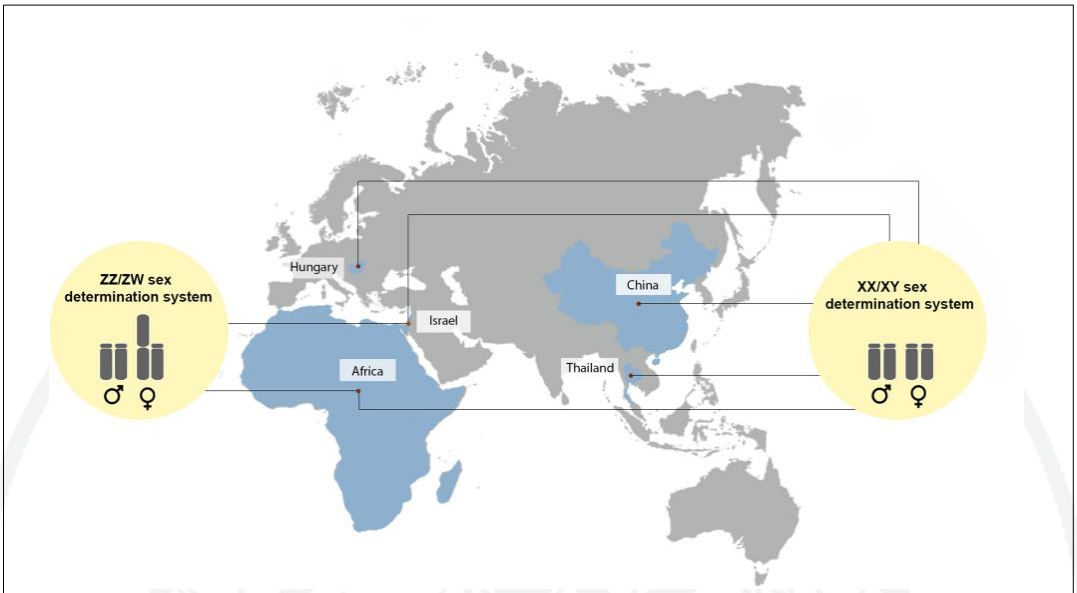

FIGURE 5 | Population map indicating a male heterogamety (XX/XY) and female heterogamety (ZZ/ZW) belonging to different groups (Ozouf-Costaz et al., 1990; Teugels et al., 1992; Liu et al., 1996; Galbusera et al., 2000; Kovács et al., 2001; Maneechot et al., 2016; Ponjarat et al., 2019).

in mammals (Mitchell et al., 1998; Uechi et al., 2002; Kuroiwa et al., 2010; Hughes et al., 2015). This could be further studied by other approaches such as whole genome sequencing and genome assembly. One moderately female-linked locus was observed in *C. gariepinus* corresponding to GGA13 and partially homologous to sex chromosomes of platypus (*Ornithorhynchus anatinus*) and W sex chromosome of Siamese cobra (*Naja kaouthia*) (Veyrunes et al., 2008; Singchat et al., 2018; **Figure 6**). Moderately sex-linked loci derived from 70:30 and 80:20 (male:female) filtering criteria indicate partial recombination between X and Y sex chromosomes. These loci are relatively close to potential male-determining regions but they are not intimately connected.

Although transposable elements can be dispersed on many chromosomes or even throughout the entire genome, large amplification of the elements are often found on sex chromosomes in many organisms (Ahmad et al., 2020; Koomgun et al., 2020; Laopichienpong et al., 2020; Yoshido et al., 2020). The presence of accumulated TEs leads to the establishment of heterochromatin, and possibly also to TE recombination between non-homologous copies. The activity of transposable elements could play an important role in sex chromosome differentiation through mechanisms of chromosomal breakage, deletion, and rearrangement (Chalopin et al., 2015; Dechaud et al., 2019). Seven out of 41 male-linked SNP/PA loci were identical to transposable elements such as *Gypsy*, *Mariner/Tc1*, and *SINE*, which are commonly distributed on sex chromosomes in Japanese rice fish (*O. latipes*) and half-smooth tongue sole (*C. semilaevis*) (Chen et al., 2014; Inoue et al., 2017).

Same Species of North African Catfish at Different Localities Show Different Sex Determination Systems

Karyotypes of *C. gariepinus* from different localities of tropical Africa and Israel are very stable and exhibit the ZZ/ZW type (Ozouf-Costaz et al., 1990; Teugels et al., 1992; Bakhoum, 1996; Okonkwo and Obiakor, 2010). However, our analysis of *C. gariepinus* with sampled populations from Thailand indicates the presence of the XX/XY co-existing with ZZ/ZW type. Possibly, *C. gariepinus* has two different sex chromosome groups in Bangui, Central African Republic, West Africa, while other fish species such as the southern platyfish, *Xiphophorus maculatus*, exhibit a different mechanism of sex determination among diverse populations (Volff and Schartl, 2001). Similarly, both ZZ/ZW and XX/XY sex chromosome systems have been reported in the glass knifefish, *Eigenmannia virescens*. The ZW group is distributed in Mato Grosso do Sul State, Brazil, with the XY group in the Tietê River, Brazil (Almeida-Toledo et al., 2001; Fernandes et al., 2019), while *Ancistrus* cf. *dubius* revealed female heterogamety in the Paraguay River Basin, Brazil and male heterogamety in the Coxipó River, Brazil (Mariotto et al., 2004; Mariotto and Miyazawa, 2006). Another well-known example of ZW/XY co-occurrence has been reported in some fish species of cichlids such as *Oreochromis niloticus*, *Oreochromis aureus*, *Astatotilapia burtoni* and *Metriaclima pyrsonotus*. These species have a male heterogametic (XY) system and a female heterogametic (ZW) system on different linkage groups (Ser

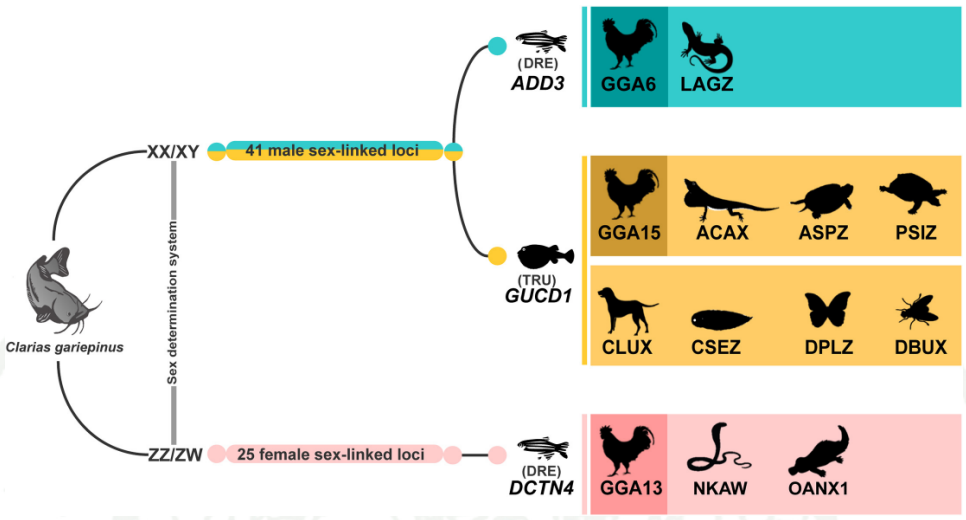

FIGURE 6 | Moderately male-linked and female-linked loci of Africa catfish (*Clarias gariepinus*) showing partial homologies with putative genes: *ADD3* (*Danio rerio*) (Pasquier et al., 2016), *GUCD1* (*Takifugu rubripes*) (Elmerot et al., 2002) and *DCTN4* (*Danio rerio*) (Bayés et al., 2017). These genes were located on chromosomes of chicken (*Gallus gallus*, GGA) and other amniote sex chromosomes, such as sand lizard (*Lacerta agilis*, LAG), green anole (*Anolis carolinensis,* ACA), spiny softshell turtle (*Apalone spinifera,* ASP), Chinese softshell turtle (*Pelodiscus sinensis,* PSI), dog (*Canis lupus familiaris,* CLU), tongue sole (*Cynoglossus semilaevis,* CSE), and invertebrates, such as fruit fly (*Drosophila busckii,* DBU), monarch butterfly (*Danaus plexippus plexippus,* DPL), and female-specific loci of African catfish (*Clarias gariepinus*) showing homologies with chicken (*Gallus gallus*, GGA), platypus (*Ornithorhynchus anatinus,* OAN), Siamese cobra (*Naja Kaouthia*, NKA). Chromosomal locations of genes in the amniotes were obtained from comparative gene mapping (chromosome mapping via a cytogenetic technique) and whole genome sequencing as the following sources: GGA from Matsuda et al. (2005), ACA from Alföldi et al. (2011), LAG from Srikulnath et al. (2014), ASP from Badenhorst et al. (2013), PSI from Kawagoshi et al. (2009), CLU from Lindblad-Toh et al. (2005), DBU from Renschler et al. (2019), CSE from Chen et al. (2014), DPL from Gu et al. (2019), OAN from Veyrunes et al. (2008), and NKA from Singchat et al. (2018).

et al., 2010; Cnaani, 2013; Moore and Roberts, 2013; Conte et al., 2017; Gammerdinger and Kocher, 2018). The majority of the sex chromosome systems in clariid catfish show the ancestral karyotype with ZZ/ZW system (Agnèse et al., 1990; Volckaert and Agnèse, 1996). This suggests that the ZW group with differentiated sex chromosomes of *C. gariepinus* might be distributed in West Africa, whereas the turnover of sex chromosome systems might have occurred in ancestral homomorphic ZW sex chromosomes, resulting in the XY group. Historically, more than one stock of *C. gariepinus* has been introduced to Thailand but the origin of this population remains unknown (Wachirachaikarn and Na-Nakorn, 2019). Thus, it is necessary to identify potential geographic variation in sex linkage across these loci, and across boundary regions between the geographic groups, as well as examining the phylogenetic history for various populations of *C. gariepinus*. Moreover, it is very interesting to examine the mutual affinity of the two different systems of sex determination when they meet secondarily after divergence from each other. Specimen collection of *C. gariepinus* from Africa with the XY group is necessary to prove these hypotheses.

A distinct difference exists between growth rates of the two sexes in *C. gariepinus* (Henken et al., 1987; Falaye et al., 2011), and improvement of genome manipulation such as gynogenesis or androgenesis and hormone treatment procedures by early molecular sexing could be economically important for species aquaculture. Results of genome-wide SNP such as those from DArTseq™ methodology provide sex-specific SNP/PA loci in moderately sex-linked loci for identifying sex determination and sex-linked markers, although this can be applied only narrowly across species (Lambert et al., 2016; Hill et al., 2018). The next phase of this study requires a PCR-based approach for sex identification to provide a genotypic tool for the practical sexing of individuals, allowing accurate detection of sex in populations through insight into the evolutionary history of the Y chromosome. However, failure of the PCR validation step has often been observed after DArTseq™ or RADseq bioinformatics analysis (Gamble et al., 2015). This outcome might result from conserved regions in both sexes of sequences adjacent to sex-specific restriction sites (Gamble, 2016).

CONCLUSION

We have presented an approach of genome-wide SNP used to identify a notable number of moderately sex-linked loci given the small portion of the genome involved. Results of moderately linked (and lacking sex-specific) markers in both male and

female individuals show that the male heterogametic XX/XY sex determination system should co-exist with ZW system in the same individuals of *C. gariepinus*, indicating a probable ongoing transition between two sex chromosome systems. However, it remains unclear whether the location of large genomic regions between X-specific and Y-specific fragments is associated with differentiation of sex chromosomes and sex-determination regions. It might be possible that the two *C. gariepinus* populations exhibit different sex chromosome systems. Thus, the possibility exists of within-species sex chromosome turnover, which could be further tested by other methods such as genome assembly. Further analysis of unrelated individuals from the wild is also required to understand the dynamics of the sex determination system in this lineage. Chromosome mapping using fluorescence *in situ* hybridization (FISH) technique on sex-specific loci should be performed on both mitotic and meiotic chromosomes, while immunostaining of synaptonemal complexes may show regions of asynapsis on putative sex chromosomes. A high-quality whole genome assembly for North African catfish will further enhance our understanding of the sex-determination mechanism and lead to the development of more robust assays for genotypic sex, allowing a much greater degree of genetic improvement in the breeding management of North African catfish, bighead catfish, and their hybrids.

DATA AVAILABILITY STATEMENT

The full dataset and metadata from this publication are available from the Dryad Digital Repository. Dataset, https://doi.org/10.5061/dryad.prr4xgxj6.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal care and all experimental procedures were approved by the Animal Experiment Committee, Kasetsart University (Approval No. ACKU61-SCI-026).

AUTHOR CONTRIBUTIONS

DN, TP, and KS drafted the manuscript and conducted the experiments. DN, NL, EK, and KS conceived the ideas and designed the methodology. DN, EK, and KS participated in the data analysis. DN, TP, JP, NL, EK, WS, SA, NM, SP, UN-N, and KS reviewed the data and the manuscript. All authors gave final approval for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2020.562856/full#supplementary-material

Supplementary Table 1 | Chromosomal locations for restriction fragment presence/absence (PA) loci of African catfish (*Clarias gariepinus*) BLAST in Japanese rice fish (*Oryzias latipes*), zebrafish (*Danio rerio*), Japanese pufferfish (*Takifugu rubripes*) and chicken (*Gallus gallus*) genomes (30:70, male:female) (ZZ/ZW sex-determination type).

Supplementary Table 2 | Gene function and pathway for restriction fragment presence/absence (PA) loci of African catfish (*Clarias gariepinus*) from a BLAST search of Japanese rice fish (*Oryzias latipes*), Japanese pufferfish (*Takifugu rubripes*), zebrafish (*Danio rerio*) and chicken (*Gallus gallus*) genome (30:70, male:female) (ZZ/ZW sex-determination type).

Supplementary Table 3 | Repeat search for restriction fragment presence/absence (PA) loci of African catfish (*Clarias gariepinus*) (30:70, male:female) (ZZ/ZW sex-determination type).

Supplementary Table 4 | Gene function and pathway for restriction fragment presence/absence (PA) loci of African catfish (*Clarias gariepinus*) from a BLAST search of Japanese rice fish (*Oryzias latipes*), Japanese pufferfish (*Takifugu rubripes*), zebrafish (*Danio rerio*) and chicken (*Gallus gallus*) genome (70:30, male:female) (XX/XY sex-determination type).

Supplementary Table 5 | Repeat search for restriction fragment presence/absence (PA) loci of African catfish (*Clarias gariepinus*) (70:30, male:female) (XX/XY sex-determination type).

Supplementary Table 6 | Chromosomal locations for restriction fragment presence/absence (PA) loci of African catfish (*Clarias gariepinus*) BLAST in Japanese rice fish (*Oryzias latipes*), zebrafish (*Danio rerio*), Japanese pufferfish (*Takifugu rubripes*) and chicken (*Gallus gallus*) genomes (70:30, male:female) (XX/XY sex-determination type).

Supplementary Table 7 | Homology for restriction fragment presence/absence (PA) loci of African catfish (*Clarias gariepinus*) from a BLAST search of Japanese rice fish (*Oryzias latipes*), Japanese pufferfish (*Takifugu rubripes*), zebrafish (*Danio rerio*) and chicken (*Gallus gallus*) genome (30:70, male:female) (ZZ/ZW sex-determination type).

Supplementary Table 8 | Homology for restriction fragment presence/absence (PA) loci of African catfish (*Clarias gariepinus*) from a BLAST search of Japanese rice fish (*Oryzias latipes*), Japanese pufferfish (*Takifugu rubripes*), zebrafish (*Danio rerio*) and chicken (*Gallus gallus*) genome (70:30, male:female) (XX/XY sex-determination type).

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Genome-wide SNP analysis suggests male heterogamety in bighead catfish (*Clarias macrocephalus*, Günther, 1864)

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ABSTRACT

Bighead catfish (*Clarias macrocephalus*, Günther, 1864) is an important aquacultural species that plays a crucial role in the economy of Southeast Asia. Crossbreeding between female bighead catfish and male African catfish (*C. gariepinus*, Burchell, 1822) is used to produce hybrids with vigorous phenotypes. However, sterility of the hybrid is a major obstacle to their mass production. There is an emerging hypothesis that the complexity of the sex-determination system between two parental species might affect sterility. Previous studies investigated the co-existence of XX/XY and ZZ/ZW sex-determination systems in the African catfish population in Thailand, but in bighead catfish the sex-determination system remains poorly understood. In this study, the sex-determination system of the bighead catfish was examined using Diversity Arrays Technology to identify the genomic variants associated with sex-linked regions. The results support the hypothesis of the previous study that the bighead catfish might exhibit a male heterogametic XX/XY sex-determination system with multiple male-linked loci. One of the male-linked loci showed homology with the *GTSF1L* gene, which shows a testis-enriched expression pattern. Two of the male-linked loci were partially homologous to transposable element. Male-linked loci on the putative Y sex chromosome were identified as an extremely small proportion of the genome. A PCR-based DNA marker was developed to validate the male-linked loci in the bighead catfish. Our findings provide novel insights into sex-determination mechanisms in clariid catfish and will contribute to genetic improvements in breeding programs.

1. Introduction

Of the 116 species of catfish classified in the family Clariidae, four species – walking catfish (*Clarias batrachus*, Linnaeus, 1758), whitespotted catfish (*C. fuscus*, Lacepède, 1803), bighead catfish (*C. macrocephalus*, Günther, 1864), and African catfish (*C. gariepinus*, Burchell, 1822) – are the most economically important in aquaculture in Asia (Adan, 2000; Na-Nakorn and Brummentt, 2009; FAO, 2020).

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Catfish aquaculture production increased globally from 137,792,918 t in 2000 to 211,906,372 t in 2018 (FAO, 2021). Historically, bighead catfish is favored for its taste and meat texture; however, its culture remains limited because of its slow growth and high sensitivity to disease (Na-Nakorn et al., 1993; Senanan et al., 2004). Crossbreeding between female bighead catfish and male African catfish has improved disease resistance, and F_1 hybrids show rapid growth (Na-Nakorn et al., 1994; Suprapto et al., 2017). As a result, a large number of hybrids are propagated in aquaculture and represent the bulk of catfish production in Thailand (Na-Nakorn, 2001). The F_1 hybrids are physically more vigorous than either parental species but their mass production has been limited by variable reproductive failure (Ponjarat et al., 2019). Previous studies have determined the diploid chromosome numbers of the African catfish and the bighead catfish to be 56 and 54, respectively, while that of the F_1 hybrid is 55 (Agnese and Teugels, 2005; Maneechot et al., 2016; Ponjarat et al., 2019). Sterile male F_1 hybrids show hybrid dysgenesis, including spermatogenic disruption, with a decreased number and/or malformation of mature sperm. This outcome might result from the failure of homologous chromosome pairing and abnormal spermatogenesis at pachytene and/or metaphase in the F_1 hybrid, leading to meiotic arrest in male hybrids (Ponjarat et al., 2019). Treatment with sex hormones, in an attempt to promote spermatogenesis, fails to overcome the sterility of male F_1 hybrids (Abol-Munafi et al., 2006). By contrast, female F_1 hybrids are fertile and can produce large numbers of backcross progeny, with both parental species showing low embryo mortality (Tumennasan et al., 1997; Na-Nakorn et al., 2004; Abol-Munafi et al., 2006). This might result from the less stringent meiotic checkpoints during oogenesis or complex interaction between two different sex-determination systems (Ponjarat et al., 2019). The homomorphic sex chromosome of the African catfish exhibits co-existence of the XX/XY and ZZ/ZW systems in Thailand (Nguyen et al., 2021), whereas the bighead catfish probably expresses the XX/XY system (Na-Nakorn, 1995). If sex determination of the bighead catfish follows the XX/XY system, breeding programs of this species and its hybrids can be improved through backcross breeding, inbreeding, selective breeding, genetic manipulation, and production of monosex individuals (Gjedrem, 1997; Senanan et al., 2004; Argue et al., 2014; Onyia et al., 2019). A proper understanding of the sex-determination system in the bighead catfish is therefore necessary.

In the *Clarias* lineage, walking catfish, mudfish (*C. anguillaris*, Linnaeus, 1758), and West African catfish (*C. ebriensis*, Pellegrin, 1920) have a ZZ/ZW sex-determination system (Pandey and Lakra, 1997; Eyo and Effiong, 2005), whereas that of white spotted clarias is XX/XY. The African catfish is the most complex species with XX/XY or ZZ/ZW systems in different geographical populations (Ozouf-Costaz et al., 1990; Teugels et al., 1992; Liu et al., 1996; Eding et al., 1997; Galbusera et al., 2000; Eyo and Effiong, 2005; Nguyen et al., 2021), or both systems in the same population (Nguyen et al., 2021). In bighead catfish, no heteromorphic sex chromosomes have been identified (Siraj et al., 2009; Maneechot et al., 2016; Ponjarat et al., 2019); however, gynogenesis processes can be induced in bighead catfish to produce all-female stocks, which is suggestive of a possible heterogametic (XX/XY) system (Na-Nakorn, 1995). In the present study, to identify and confirm the sex-determination system of the bighead catfish, we performed genome-wide single-nucleotide polymorphism (SNP) analysis using Diversity Arrays Technology (DArTseq™) of captive-bred individuals scored with phenotypic sex. This method generated loci assorted to the particular sex by identifying loci linked to sex-determining regions of the genome. A molecular marker was developed to validate the sex-linked SNP pool. The results provide insights to improve the understanding of sex-determination systems in clariid catfish and could contribute to genetic improvements in aquaculture breeding programs.

2. Materials and methods

2.1. Specimens and DNA extraction

Fifteen male and 15 female bighead catfish individuals of standard weight 1.7–2.0 kg and length 35–40 cm were sampled. The individuals were selected randomly from a large breeding stock (Kasetsart University, Bangkok, Thailand) to avoid the possibility of a high incidence of siblings in our genetic pool. Animal care and all experimental procedures were approved by the Animal Experiment Committee, Kasetsart University, Thailand (Approval no. ACKU61-SCI-026) and conducted in accordance with the Regulations on Animal Experiments at Kasetsart University. The sex of each individual was determined from external morphology and internal examination of gonadal morphology (Kitano et al., 2007; Esmaeili et al., 2017; Ponjarat et al., 2019). The fins were removed for DNA extraction. Total genomic DNA was extracted following the standard salting-out protocol as described previously with slight modification for different tissues (Supikamolseni et al., 2015). The quality of extracted DNA was evaluated by gel electrophoresis, and samples with high-molecular-weight DNA were stored at −20 °C until required for DArTseq™ library construction as described previously (Koomgun et al., 2020; Laopichienpong et al., 2021; Nguyen et al., 2021).

2.2. DArT sequencing and genotyping

The DArTseq™ methodology followed Jaccoud et al. (2001). Genotyping of multiple loci was performed using DArTseq™ (Diversity Arrays Technology Pty Ltd., Canberra, ACT, Australia) for SNP loci and *in silico* DArT (variability in SNP loci generates presence/absence polymorphism in restriction sites, so-called PA markers) to determine the candidate sex-specific loci between male and female individuals. Approximately 100 ng DNA from each sample was used for development of the DArTseq™ arrays. The DNA samples were subjected to digestion/ligation reactions as described previously (Kilian et al., 2012; Koomgun et al., 2020; Laopichienpong et al., 2021; Nguyen et al., 2021). Sequences were processed using proprietary DArTseq™ analytical pipelines (Ren et al., 2015). Outputs generated by DArTsoft14 were filtered based on reproducibility values, average count for each sequence (sequencing depth), balance of average counts for each SNP allele, and call-rate (proportion of samples for which the marker was scored) as described previously (Koomgun et al., 2020; Laopichienpong et al., 2021; Nguyen et al., 2021).

2.3. Marker selection and DArT sequencing analysis

Sex-linked or -specific loci were obtained from the analysis of SNP co-dominant markers and PA dominant markers. For an XX/XY sex-determination system, the SNP and PA loci sequenced for 70%, 80%, 90%, and 100% of males were included in a separate data set. Loci that passed the 100% filtering criterion were designated as perfectly sex-linked, whereas those passing at 70%–90% were considered moderately sex-linked loci as described previously (Koomgun et al., 2020; Laopichienpong et al., 2021; Nguyen et al., 2021). An opposite but similar approach was conducted to target loci with a ZZ/ZW system. The Hamming distance was calculated to determine the number of combined loci between male and female individuals for pairwise differences in SNP and PA loci using the "rdist" function in R version 3.5.1. Heatmaps were generated using the ggplot2 package for R (R Core Team, 2019). The Hamming distance, Cochran–Armitage trend test (CATT), and polymorphism information content (PIC), as an index for evaluation of the informativeness of SNP and PA loci, were calculated as described previously (R Core Team, 2019; Sven and Klaus, 2019; Koomgun et al., 2020; Laopichienpong et al., 2021; Nguyen et al., 2021). The probability of candidate sex-linked loci showing random associations with sex under 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 (Koomgun et al., 2020; Nguyen et al., 2021).

2.4. Comparison of potential sex-linked loci

For all candidate loci, the sex-linked loci under the criteria 90:10, 80:20, and 70:30 were designated as moderately sex-linked loci. Significant differences among the three groups of loci were examined using the chi-square test and the Kruskal–Wallis test using the stats R package for PA loci and the Nemenyi test for SNP loci implemented in the PMCMR package for R (R Core Team, 2019), based on the mean heterozygosity and standard deviation for each. All candidate loci were plotted against each individual using the "glPlot" function in the dartR R package. Principal coordinate analysis was performed to visualize the relatedness between males and females based on all groups of moderately sex-linked loci (Gruber and Georges, 2019; R Core Team, 2019).

2.5. Homology searches

For all sex-linked loci that met our criteria and showed a statistically significant association with phenotypic sex, a Basic Local Alignment Search Tool (BLAST) search was performed to investigate the homologies of the sex-linked SNP/PA loci against a selection of available teleost fish genomes: Japanese rice fish (*Oryzias latipes*, Temminck and Schlegel, 1850; accession no. GCA_002234675.1; Kasahara et al., 2007), zebrafish (*Danio rerio*, Hamilton, 1822; accession no. GCA_000002035.4; Broughton et al., 2001), Japanese pufferfish (*Takifugu rubripes*, Temminck and Schlegel, 1850; accession no. GCA_901000725.2; Elmerot et al., 2002), channel catfish (*Ictalurus punctatus*, Rafinesque, 1818; accession no. GCA_001660625.1; Liu et al., 2016), and an amniote reference genome (chicken, *Gallus gallus*, Linnaeus, 1758; accession no. GCA_000002315.5; International Chicken Genome Sequencing Consortium, 2004). We selected the aforementioned species as representative reference genomes in the homology search analysis due to the availability of high-quality gene annotations and almost complete up-to-date assemblies (Takeda, 2008; Zhou, 2012; Howe et al., 2013; Warren et al., 2017). The BLAST homology search was performed in two rounds. First, we aligned sex-linked loci against the reference teleost genome, and secondly we mapped the homologous genes to clarify further their location on sex chromosomes of high-quality annotated genomes representative vertebrate species. Using the BLASTn program, sex-linked loci were used to search the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and RepBase version 19.11 (Bao et al., 2015; Genetic Information Research Institute; https://www.girinst.org/). This is a specialized nucleotide sequence collection for repeated or other significant sequences, which only reports E-values lower than 0.005 and queries coverage with similarity of more than 60%.

2.6. Development of sex-linked markers

To develop sex-linked markers, the candidate male-linked or male-specific loci were selected to develop sex-linked PCR-based markers. Six and seven SNP and PA loci, respectively, were selected randomly and filtered using four criteria: no repeated sequences, one of two alleles in accordance with the base in reference, SNP-free within the flanking sequence, and flanking sequence ≥50 bp, following the method of Laopichienpong et al. (2021). Applicability of the sex-linked PCR assays was further tested and validated using all 30 bighead catfish specimens as mentioned above. The PCR amplification was performed using 7.5 μl of 2× PCR buffer KOD FX Neo, 2 mM dNTPs, 0.5 μM specific primers (Table 1), 1.0 U KOD FX Neo (Toyobo Co., Ltd., Japan), and 25 ng genomic DNA. The PCR protocol was as follows: initial denaturation at 98 °C for 5 min, followed by 35 cycles of 98 °C for 20 s, 58 °C for 45 s, and 68 °C for 2 min, and final extension at 68 °C for 5 min. The PCR products were visualized by electrophoresis in 1% agarose gel. The *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) gene was used as a positive PCR control marker using primers designed based on the conserved sequence of three teleosts (channel catfish, zebrafish, and maraena whitefish *Coregonus maraena*, Bloch, 1779) to verify that the absence of PCR products in females was not caused by failure of the PCR reactions (Ponjarat et al., 2019). To determine the conservation of sex-linked loci, all sex-linked PCR markers were applied to 30 African catfish individuals, with genomic DNA derived from our previous study (Nguyen et al., 2021). The PCR reaction was performed as mentioned above.

Table 1
Primers used for development of sex-linked restriction fragment presence/absence markers.

Primer	Sequence (5′–3′)	Size (bp)
PA35641624_F	TGCAGGCTGCCACCTATACA	208
PA35641624_R	GCTGCTGCATGACCTTAACA	
GapDH_F	TCTTATGAGCACTGTCCATGCC	101
GapDH_R	TAATGTTCTGGCTGGCACCAC	

3. Results

3.1. Determination of sex-linked loci in bighead catfish

We sequenced 15,315 SNP loci and 12,311 PA loci. The PIC values ranged from 0.37 to 0.50 for all loci, which indicates that the overall distribution of PIC values was asymmetrical and skewed toward the higher values. To determine whether XX/XY or ZZ/ZW sex determination was exhibited in bighead catfish, we compared a number of SNP and PA loci after filtering with a gradually varying set of criteria. For the ZZ/ZW type, filtering using the criterion of 30:70 male:female resulted in 31 SNP loci and 54 PA loci as moderately female-linked. Proportional pairwise Hamming distances between males and females using the moderately sex-linked SNP and PA loci showed within-sex distances of 0.427 ± 0.013 in males and 0.403 ± 0.014 in females for SNP loci, and 0.383 ± 0.013 in males and 0.367 ± 0.012 in females for PA loci. Between-sex distances were 0.713 ± 0.008 for SNP loci and 0.669 ± 0.008 for PA loci. The CATT results verified a significant association with phenotypic sex for 30 SNP loci ($\chi^2 = 2.3$–16.4, $p < 0.001$) and 54 PA loci ($\chi^2 = 5.8$–16.1, $p < 0.001$). The criterion of 20:80 male:female resulted in designation of seven SNP loci and seven PA loci as moderately female-linked. Proportional pairwise Hamming distances between males and females using the moderately sex-linked SNP and PA loci (under the null exclusive model) showed within-sex distances of 0.408 ± 0.026 in males and 0.354 ± 0.021 in females for SNP loci, and 0.290 ± 0.017 in males and 0.302 ± 0.020 in females for PA loci. Between-sex distances were 0.764 ± 0.013 for SNP loci and 0.729 ± 0.013 for PA loci. The CATT verified a significant association with phenotypic sex for seven SNP loci ($\chi^2 = 10.8$–16.4, $p < 0.001$) and seven PA loci ($\chi^2 = 10.8$–16.1, $p < 0.001$; Fig. 1). After filtering using the criteria 10:90 and 0:100 male:female, no significant SNP and PA loci were detected for sex-linked loci (Fig. 2, Table 2). Chi-square tests show that the 30:70 and 20:80 filtering criteria indicated no significant differences in males ($\chi^2 = 5.230 \times 10^{-32}$, $p = 1$) and females ($\chi^2 = 4.659 \times 10^{-32}$, $p = 1$) for PA loci. Kruskal–Wallis tests show that these filtering criteria produced no significantly different percentages of heterozygosity in males ($H = 1.23$, $p = 0.267$) and females ($H = 0.89$, $p = 0.346$) for SNP loci (Fig. S1). Pairwise comparisons using the Nemenyi test with chi-square approximation for independent samples reveal that the 30:70 and 20:80 filtering criteria resulted in no significant difference in heterozygosity compared with the other filters for males ($p = 0.15$) and females ($p = 0.24$).

By contrast, for the XX/XY type, filtering using the criterion of 70:30 male:female yielded 49 SNP loci and 72 PA loci that were designated

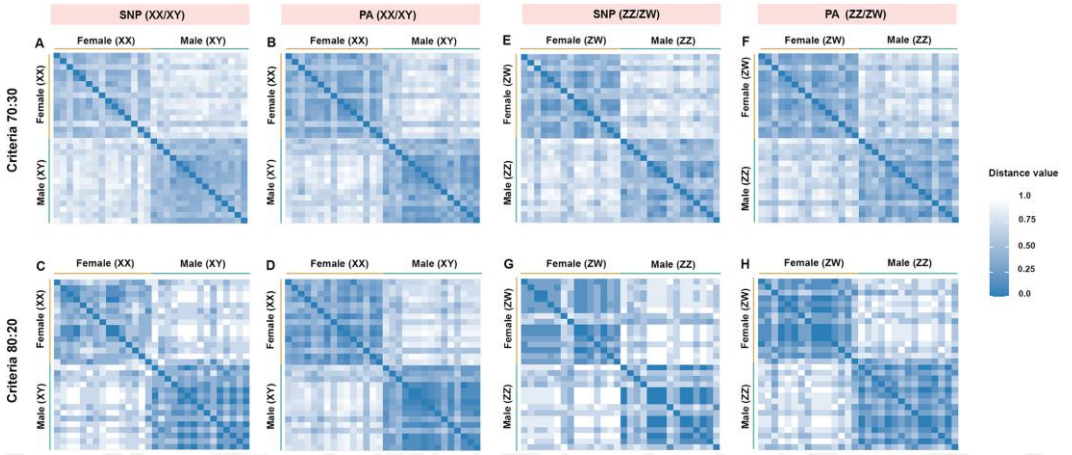

Fig. 1. Hamming distances between male and female individuals of bighead catfish (*Clarias macrocephalus*, Günther, 1864). The loci were filtered under different criteria. (A) Single- nucleotide polymorphic (SNP) loci filtered under the criterion 70:30 (male:female), (B) Restriction fragment presence/absence (PA) loci under the criterion 70:30 (male:female), (C) SNP loci under the criterion 80:20 (male:female), (D) PA loci under the criterion 80:20 (male:female), (E) SNP loci under the criterion 30:70 (male:female), (F) PA loci under the criterion 30:70 (male:female), (G) SNP loci under the criterion 20:80 (male:female), and (H) PA loci under the criterion 20:80 (male:female).

male-linked. Proportional pairwise Hamming distances between male and female bighead catfish using sex-linked SNP and PA loci (under the null exclusive model) show lower within-sex distances of 0.401 ± 0.009 in males and 0.482 ± 0.012 in females for SNP loci, and 0.347 ± 0.011 in males and 0.379 ± 0.012 in females for PA loci. Between-sex distances of 0.698 ± 0.006 for SNP loci and 0.676 ± 0.007 for PA loci were observed. CATT results verified significant associations with phenotypic sex for 49 SNP loci ($\chi^2 = 4.3$–21.7, $p < 0.001$) and 72 PA loci ($\chi^2 = 5.8$–22.9, $p < 0.001$). Seven SNP loci and 17 PA loci were associated with males based on the criterion of 80:20 male:female. Proportional pairwise Hamming distances between male and female bighead catfish using sex-linked SNP and PA loci showed lower within-sex distances 0.320 ± 0.018 in males and 0.450 ± 0.019 in females for SNP loci, and 0.263 ± 0.016 in males and 0.323 ± 0.014 in females for PA loci. Between-sex distances of 0.754 ± 0.012 for SNP loci and 0.737 ± 0.009 for PA loci were observed. CATT results verified significant associations with phenotypic sex for seven SNP loci ($\chi^2 = 10.8$–21.0, $p < 0.001$) and 17 PA loci ($\chi^2 = 10.8$–20.0, $p < 0.001$; Fig. 1). After filtering with the criteria 90:10 and 100:0 male:female, no significant sex-linked SNP or PA loci were observed (Fig. 2, Table 3). Chi-square tests show that the 70:30 and 80:20 filtering criteria indicated no significant differences in males ($\chi^2 = 1.893 \times 10^{-32}$, $p = 1$) and females ($\chi^2 = 6.685 \times 10^{-32}$, $p = 1$) for PA loci. Kruskal–Wallis tests show that these filtering criteria yielded no significantly different percentages of heterozygosity in males ($H = 1.29$, $p = 0.256$) and females ($H = 0.692$, $p = 0.406$) for SNP loci (Fig. S1). Pairwise comparisons using the Nemenyi test with chi-square approximation for independent samples reveal that the 70:30 and 80:20 filtering criteria resulted in no significant difference in heterozygosity compared with the other filters for males ($p = 0.48$) and females ($p = 0.59$). A glPlot revealed that the sample group showed similarity between sexes when moderately sex-linked loci were considered in both XX/XY and ZZ/ZW sex-determination systems (Fig. 3).

For a range of sample sizes and loci, a sample of 30 phenotypically sexed individuals is essential to minimize the probability of selecting less than one spurious sex-linked marker. The P_i probability that a single locus exhibited a sex-linked pattern by chance was 9.31×10^{-10}. Considering that we analyzed the full data set (no filtering) of 27,626 loci (including SNP and PA loci), this probability translates into an expected number of perfect sex-linked loci according to chance alone of 2.57×10^{-5}.

3.2. Homology of putative sex-linked loci

With regard to the ZZ/ZW sex-determination system, female sex-linked loci of bighead catfish showed sequence homology with the Japanese rice fish, Japanese puffer, zebrafish, channel catfish, and chicken genomes on the basis of global BLAST analyses of NCBI databases (Table S1). Eight of the 84 SNP and PA loci were homologous with the putative genes *RUNX2* (runt-related transcription factor 2), *PHKA1* (phosphorylase kinase regulatory subunit alpha 1), *CNNM4* (metal transporter CNNM4), *ABI2* (Abl interactor 2), *GFI1* (zinc finger protein Gfi-1), *ATG9A* (Autophagy-related protein 9A), *RTN4RL2* (reticulon 4 receptor-like 2), and *ROBO4* (roundabout guidance receptor 4; Table S2). Moreover, four SNP loci and nine PA loci of the 84 loci show partial homology with transposable elements (TEs), consisting of three loci with similarity to Tc1/mariner families, one locus to a Kolobok element (a superfamily of DNA transposons), seven loci to Gypsy families, one locus to short interspersed nuclear elements, and one locus to chicken repeat 1 (Table S3). One locus filtered under the criterion 20:80 male:female was identified as a Gypsy family member (Table S4). By contrast, with regard to the XX/XY sex-determination system, male sex-linked loci of the bighead catfish show sequence homology with the Japanese rice fish, Japanese puffer, zebrafish, channel catfish, and chicken genomes on the basis of global BLAST analyses of NCBI databases (Table S5). Sixteen of 121 SNP and PA loci were homologous with the putative genes *GOLGA4* (golgin subfamily A member 4), *ARFRP1* (ADP ribosylation factor related protein 1), *NSMCE2* (E3 SUMO–protein ligase NSE2), *FGFRK1* (fibroblast growth factor receptor–like 1), *DNAH7* (dynein heavy chain 7, axonemal), *SMARCAD1* (SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A containing DEAD/H box 1), *LMBRD2* (G-protein coupled receptor-associated protein LMBRD2), *CNOT4* (CCR4-NOT transcription complex subunit 4-like), *CHD3* (chromodomain-helicase-DNA-binding protein 3), *TFAP2B* (transcription factor AP-2-beta), *WDR43* (WD repeat-containing protein 43), *ETV6* (transcription factor ETV6), *MED8* (mediator of RNA polymerase II transcription subunit 8), *TRAM1L1*

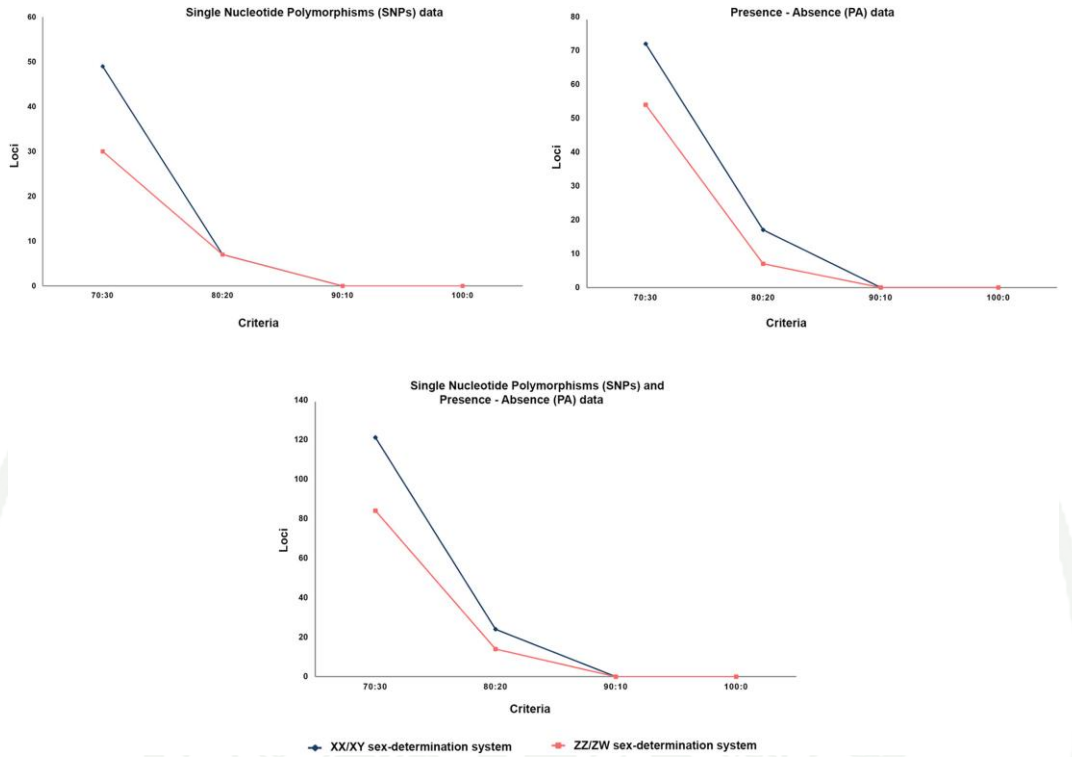

Fig. 2. Number of loci in different hypothetical sex-determination systems after filtering under different criteria. The *y*-axis is number of loci and the *x*-axis is the filter criteria. Sex-linked sequences are shown at the bottom of the figure. The female sex-determination system is indicated in red and the male sex-determination system in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
DArT analysis for 15 male and 15 female individuals of bighead catfish (*Clarias macrocephalus*, Günther, 1864; ZZ/ZW sex-determination type).

	30:70 male:female		20:80 male:female		10:90 male:female		0:100 male:female	
	SNP[a]	PA[b]	SNP[a]	PA[b]	SNP[a]	PA[b]	SNP[a]	PA[b]
Total number of DArT analyses	15,315	12,311	15,315	12,311	–	–	–	–
Sex-specific loci	30	54	7	7	–	–	–	–
Overall mean distance between males and females	0.713 ± 0.008	0.669 ± 0.008	0.764 ± 0.013	0.729 ± 0.013	–	–	–	–
Overall mean distance within females	0.403 ± 0.014	0.367 ± 0.012	0.354 ± 0.021	0.302 ± 0.02	–	–	–	–
Overall mean distance within males	0.427 ± 0.013	0.383 ± 0.013	0.408 ± 0.026	0.290 ± 0.017	–	–	–	–

[a] SNP, single-nucleotide polymorphic loci.

[b] PA, Restriction fragment presence/absence loci.

(translocating chain-associated membrane protein 1-like 1), *PCDH12* (protocadherin-12), and *GTSF1L* (gametocyte-specific factor 1-like; Table S6). Twelve SNP loci and 12 PA loci filtered under the criterion 70:30 male:female show partial homology with TEs consisting of 11 loci to Tc1/mariner families, one locus to the hAT transposon superfamily, one locus to Helitron, one locus to Zisupton, one locus to the transposon piggyBac, five loci to Gypsy families, one locus to short interspersed nuclear elements, one locus to an L2 clade of non-LTR retrotransposons, one locus to the retroelement *Rex1*, and one locus to the endogenous retroviruses 2 superfamily (Tables S7 and S8). One PA locus of the bighead catfish was homologous to the genomic region of the chicken Z chromosome (Table S5).

3.3. PCR-based approach for validation of sex-specific loci

A PCR-based method was applied to validate the sex-linked loci in bighead catfish. One of the 13 PA loci (locus id: 35641624) was validated (Table 1). Only male individuals were identified by a single 208 bp DNA band for the PA35641624 marker (locus id: 35641624), and no specific DNA band was identified in females (Fig. 4). Partial fragments of the *GAPDH* gene were amplified in all males and females as a positive single 101 bp DNA band in order to examine the failure to amplify the

Table 3
DArT analysis for 15 male and 15 female individuals of bighead catfish (*Clarias macrocephalus*, Günther, 1864; XX/XY sex-determination type).

	70:30 male:female		80:20 male:female		90:10 male:female		100:0 male:female	
	SNP[a]	PA[b]	SNP[a]	PA[b]	SNP[a]	PA[b]	SNP[a]	PA[b]
Total number of DArT analyses	15,315	12,311	15,315	12,311	–	–	–	–
Sex-specific loci	49	72	7	17	–	–	–	–
Overall mean distance between males and females	0.698 ± 0.006	0.676 ± 0.007	0.754 ± 0.012	0.737 ± 0.009	–	–	–	–
Overall mean distance within females	0.482 ± 0.012	0.379 ± 0.012	0.450 ± 0.019	0.323 ± 0.014	–	–	–	–
Overall mean distance within males	0.401 ± 0.009	0.347 ± 0.011	0.320 ± 0.018	0.263 ± 0.016	–	–	–	–

[a] SNP, single-nucleotide polymorphic loci.

[b] PA, Restriction fragment presence/absence loci.

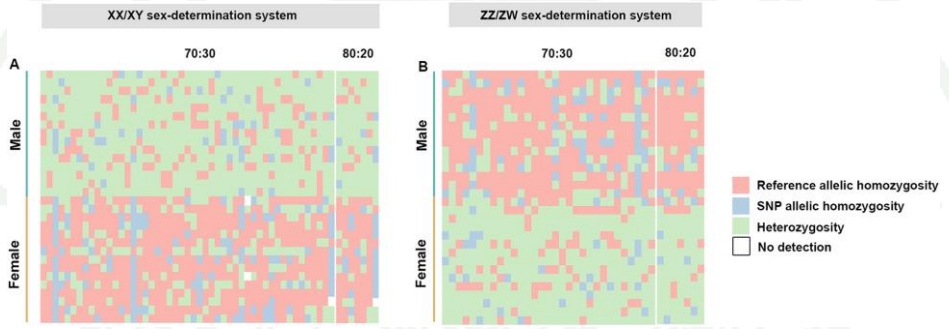

Fig. 3. (A) Index of 121 sex-linked loci filtered under the criterion 70:30 (male:female; XX/XY sex-determination system), and (B) index of 84 sex-linked loci filtered under the criterion 30:70 (male:female; ZZ/ZW sex-determination system). The plots were generated using the "glPolt" function in the R package dartR (Gruber and Georges, 2019; R Core Team, 2019). Pink shading indicates reference allelic homozygosity, green is indicative of heterozygosity, and blue indicates SNP allelic homozygosity. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

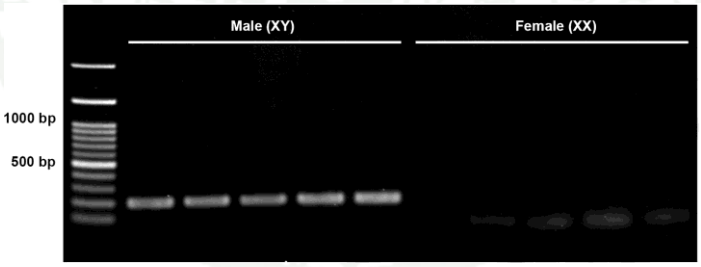

Fig. 4. Agarose gel electrophoresis of PCR products of the validation test of sex-linked loci in male and female individuals of bighead catfish (*Clarias macrocephalus*, Günther, 1864). Molecular size of DNA is indicated in the left lane using OneMARK B ladder (Bio-Helix Co., Ltd., Taipei, Taiwan).

sex-linked loci from primer sets (data not shown). To determine the conservation of sex-linked loci, all the sex-linked PCR markers were applied to African catfish, and a single 208 bp DNA band was detected in both males and females (Fig. S2).

4. Discussion

Hybridization of parental fish that differ in their chromosome number can produce fertile hybrids, as observed for North African catfish (*C. gariepinus*, $2n = 56$) × Vundu catfish (*Heterobranchus longifilis*, Valenciennes, 1840, $2n = 52$; Legendre et al., 1992). The F_1 hybrids between female bighead catfish and male African catfish exhibit rapid growth and high resistance to disease, but their mass production is limited by reproductive failure (Ponjarat et al., 2019). To increase the hybrid broodstock, large numbers of female bighead catfishes, native to Southeast Asia, are required annually to produce the hybrid; however, the captive population of the pure line is limited, and the wild population has declined as a result of urbanization and contamination of the hybrid in the wild (Senanan et al., 2004; Duong et al., 2017). Only female hybrids are fertile, with the potential to produce large numbers of backcross progeny that are fertile and showed low embryo mortality (Na-Nakorn et al., 2004; Abol-Munafi et al., 2006). Thus, a comprehensive understanding of sex determination in the African catfish and bighead catfish is important. The diploid gynogenesis in bighead catfish produces all females, suggesting XX/XY sex-determination (Na-Nakorn, 1995). In the present study, DArTseq™ technology was applied to a

large number of SNP and PA loci to enable prediction of sex-linked loci for bighead catfish using a sample size of 30 individuals (15 males and 15 females). We identified 24 SNP or PA markers for male-linked loci and 14 SNP or PA markers for female-linked loci with the criterion 80:20 male:female across all specimens examined. Although many false-positive signals might be expected from such specimens due to their more diverse genetic background (Gamble et al., 2015), the present data show the feasibility of the approach. The probability that one locus will show sex linkage spuriously was 9.31×10^{-10} from the full data set (without filtering) of 27,626 loci (including SNP and PA loci), whereas the expected amount of sex linkage was estimated to be 2.57×10^{-5}. Identification of any sex-linked loci by chance thus seems unlikely. The present results indicate that the sex determination of bighead catfish tends to be the XX/XY system.

Homology searches of male-linked loci reveal that two of the 24 SNP or PA male-linked loci showed homology with partial sex chromosomes in Z chromosome of the western terrestrial garter snake (*Thamnophis elegans*) and chicken (*Gallus gallus*; Tables S5 and S9), whereas no female-linked SNP or PA loci were matched. Genomic convergence has also been detected by comparative genomics studies in which unrelated sex chromosomes share sex chromosomal linkage homologies across distantly related species (Srikulnath et al., 2009a, 2009b, 2013, 2014, 2015; Ezaz et al., 2017; Singchat et al., 2018, 2020a, 2020b; Ahmad et al., 2020; Koomgun et al., 2020; Laopichienpong et al., 2021; Nguyen et al., 2021). It is likely that convergent evolution of sex chromosomes across distantly related taxa has led to genomic elements that are particularly efficient in a sex-determination role (Singchat et al., 2020c). Interestingly, the PA35631435 locus showed homology with the *GTSF1L* gene, which shows a testis-enriched expression pattern in comparison with 26 other human tissues (Djureinovic et al., 2014). These findings suggest collectively the presence of an XX/XY sex-determination system in bighead catfish, supporting the results of diploid gynogenesis with all females (Na-Nakorn, 1995). However, we have no direct evidence that this gene is involved in sex determination for teleosts or other vertebrates, and additional information from genomic analysis and transcriptomic activity of teleosts is required to test this hypothesis. Moreover, two of the 24 male-linked SNP or PA loci filtered under the criterion 80:20 male:female were identical to TEs, such as Tc1/mariner and Zisupton, which are frequently distributed on sex chromosomes in half-smooth tongue sole (*Cynoglossus semilaevis*, Günther, 1873), southern platyfish (*Xiphophorus maculatus*, Günther, 1866), *Apareiodon* sp., canivete (*Parodon hilarii*, Reinhardt, 1867), and pongo characin (*P. pongoensis*, Allen, 1942; Böhne et al., 2012; Zhou, 2012; Chen et al., 2014; Schemberger et al., 2016). Only one female-linked SNP locus filtered under the criterion 20:80 male:female was homologous to Gypsy families. Transposable elements can be dispersed on many chromosomes or throughout the entire genome, and amplification of the elements is often observed on sex chromosomes in many organisms (Chalopin et al., 2015; Schemberger et al., 2016; Ahmad et al., 2020; Carducci et al., 2020; Singchat et al., 2020b; Koomgun et al., 2020; Yoshido et al., 2020; Laopichienpong et al., 2021). Activity of TEs may play an important role in sex-chromosome differentiation through mechanisms of chromosomal rearrangement or heterochromatin propagation as a variegated type of position effect (Chalopin et al., 2015; Dechaud et al., 2019). Moderately sex-linked loci filtered under the 70:30 and 80:20 (male:female) criteria indicate partial recombination between X and Y sex chromosomes. These loci are relatively close to potential male-determining regions but not intimately connected.

Of the 24 SNP or PA sex-specific loci discovered independently in bighead catfish, one of the 13 male-linked PA loci was validated. A non-specific band was detected in one female individual of bighead catfish, possibly connected with the stability of the primer binding site. Similar results were observed in Siamese cobra (*Naja kaouthia*, Lesson, 1831), pygmy cormorant (*Phalacrocorax pygmeus*, Pallas, 1733), great cormorant (*P. carbo*, Linnaeus, 1758), and European shag (*P. aristotelis*, Linnaeus, 1761), which exhibit heteromorphic sex chromosomes (Thanou et al., 2013; Laopichienpong et al., 2021). The remaining 12 male-linked PA markers, detected only in a portion of the samples, were weakly sex-linked among the adult samples and might be less reliable as sex-linked markers (data not shown). Failure of the PCR validation step has often been observed after DArTseq™ or RADseq bioinformatics analysis (Gamble et al., 2015; Jiang et al., 2020; Laopichienpong et al., 2021). This outcome might result from conserved regions in both sexes of sequences adjacent to sex-specific restriction sites (Gamble, 2016). The methods of polymerase chain reaction-restriction fragment length polymorphism or melting curve analysis, which offer more sensitive detection, might be alternative methods to use for the validation step. The validated male-linked PA marker was amplified and determined the sex specifically in bighead catfish but could not differentiate between male and female individuals in African catfish. Cryptic intra-chromosomal rearrangements or mutations on sex chromosomes may have occurred between two catfish species (the African catfish and the bighead catfish) after divergence. Similar phenomena have been observed in bighead carp (*Hypophthalmichthys nobilis*, Richardson, 1845), silver carp (*H. molitrix*, Valenciennes, 1844), mud crab species (*Scylla paramamosain*, Estampador, 1950; *S. tranquebarica*, Fabricius, 1798; and *S. serrata*, Forsskål, 1755), Siamese cobra, and Indochinese spitting cobra (*N. siamensis*, Laurenti, 1768; Liu et al., 2018; Shi et al., 2018; Laopichienpong et al., 2021). Analysis of additional catfish species are required to examine the presence of markers to test this hypothesis.

Both XX/XY and ZZ/ZW sex-linked loci were observed occasionally in the same bighead catfish individual (Table S10). If recombination occurs frequently in the region of the homomorphic sex chromosomes, female heterozygous sex-linked loci may not always indicate female heterogametic sex determination. By contrast, the X-linked gene, could be homologous to the male-linked marker in other species and indicate a different sex determination system. *DMRT1* is an ancient sex determining gene, found first in invertebrates (Ferguson-Smith, 2007). The bird Z chromosome includes the highly conserved *DMRT1* gene; however, a duplicated copy of *DMRT1* was identified on the medaka Y sex chromosome (Nanda et al., 1999; Matsuda et al., 2002). Moreover, *DMRT1* is a putative male determination gene in *Cynoglossus semilaevis* and the *DMRT1* gene is also a candidate sex determination gene on the Y chromosome in *Scatophagus argus* (Cui et al., 2017; Mustapha et al., 2018). Another possible explanation for this is the coexistence of the XX/XY and ZZ/ZW systems in this species. This might represent evidence of sex-chromosome turnover, which is often observed in teleosts (Myosho et al., 2015; Roco et al., 2015; Baroiller and D'Cotta, 2016). Our previous analysis of the African catfish indicates the presence of XX/XY co-existent with the ZZ/ZW type in sampled populations from Thailand (Nguyen et al., 2021). Similar cases were observed in the southern platyfish and the glass knifefish (*Eigenmannia virescens*, Valenciennes, 1836; Almeida-Toledo et al., 2001; Volff and Schartl, 2001; Mariotto et al., 2004; Mariotto and Miyazawa, 2006; Fernandes et al., 2019). An additional well-known example of ZW/XY co-occurrence is certain species of cichlids, such as Nile tilapia (*Oreochromis niloticus*, Linnaeus, 1758), blue tilapia (*O. aureus*, Steindachner, 1864), Burton's mouth-brooder (*Astatotilapia burtoni*, Günther, 1894), and zebra red dorsal (*Metriaclima pyrsonotos*, Stauffer Jr. et al., 1997). These species have a male heterogametic (XY) system and a female heterogametic (ZW) system in different linkage groups (Ser et al., 2010; Cnaani, 2013; Moore and Roberts, 2013; Conte et al., 2017; Gammerdinger and Kocher, 2018). In the present study, based on comparison among several teleost reference genomes, sex-linked loci of bighead catfish were not all located in the same linkage group. Teleost sex chromosomes are often homomorphic (*i.e.*, cytologically indistinguishable), which is problematic for genome-wide SNP analysis (Otake et al., 2010; Gammerdinger and Kocher, 2018). However, this seems to facilitate frequent sex-chromosome turnover (Gammerdinger and Kocher, 2018; Ieda et al., 2018). Variation in fish sex-chromosome systems has been recorded among closely related species or between different

populations of the same species (Takehana et al., 2008; Ross et al., 2009; Ser et al., 2010; Cnaani, 2013). In the clariid catfish lineage, most sex-chromosome systems are of the ancestral type, *i.e.*, the ZZ/ZW system (Agnèse et al., 1990; Volckaert and Agnèse, 1996; Fig. 5). Nguyen et al. (2021) asserted that the ZW group with differentiated sex chromosomes of the African catfish might be distributed in different continents, whereas the turnover of sex-chromosome systems might have occurred in ancestral homomorphic ZW sex chromosomes, which resulted in the XY group on a different continent. Compared with the African catfish, the bighead catfish is phylogenetically more primitive. These two species diverged approximately 12 million years ago (Agnese and Teugels, 2005; Hedges et al., 2015). Turnover of sex-chromosome systems may have occurred in ancestral homomorphic ZZ/ZW sex chromosomes and resulted in the proto XX/XY group in this lineage. Sex chromosomes of the bighead catfish have undergone early state differentiation, resulting in the presence of cryptic sex chromosomes. To test these hypotheses, it is necessary to identify potential populational variation in sex linkage across these loci. The possibility exists of within-species sex-chromosome turnover, which could be further tested using other methods such as genome assembly. However, the influence of noise from high-throughput sequencing technologies and/or random biological variation/association cannot be ruled out, especially with small sample sizes that might show sex-linked loci outside the sex-determination regions or even in autosomes (Gamble et al., 2017).

Sex determination of the African catfish and bighead catfish is important to improve genomic manipulation of processes such as gynogenesis or androgenesis. Hormone treatment procedures following early molecular sexing may be economically important for species aquaculture. The next phase of this study requires development of additional PCR-based markers for sex identification to provide a genotypic tool for practical sexing of individuals, which will allow accurate detection of sex in populations through insight into the evolutionary history of the Y chromosome. In addition, gene interaction and transmission mechanisms of sex determination and sex-linked loci from parental species to the F_1 hybrid require further investigation.

5. Conclusions

We identified male-linked loci on the putative Y sex chromosome of the bighead catfish using DArTseq™ technology given the small portion of the genome. The results suggest the presence of XX/XY sex determination. One male-linked locus shows homology with the *GTSF1L* gene, which revealed a testis-enriched expression pattern, whereas one other male-linked locus shows partial homology with Z sex chromosomes of the western terrestrial garter snake and chicken. The PCR-based markers, derived from male-linked loci in bighead catfish, provide information on the putative Y sex chromosome, which represents an archetype in the understanding of sex-linked loci on homomorphic X and Y sex chromosomes. Whether or not the location of large genomic regions between X- and Y-specific fragments is associated with the differentiation of sex chromosomes and sex-determination regions remains unclear. A population based study with a relatively even ratio of sexes or various bighead catfish populations from the wild and captivity is required to minimize the probability of false positives. The number of samples needed may also depend on the amount of polymorphism present in a population. Chromosome mapping of sex-linked loci on

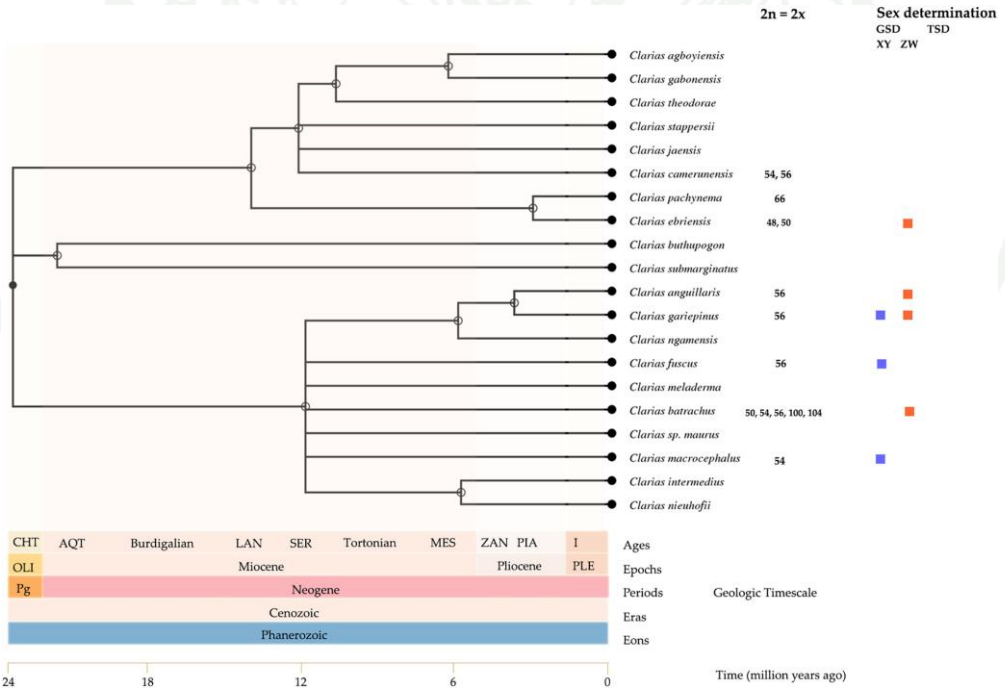

Fig. 5. Phylogenetic tree depicting species relationships and timing of diversification in the genus *Clarias* together with the number of chromosomes and sex-determination system of the species where known (Ozouf-Costaz et al., 1990; Teugels et al., 1992; Na-Nakorn, 1995; Bakhoum, 1996; Liu et al., 1996; Eding et al., 1997; Pandey and Lakra, 1997; Kovács et al., 2001; Agnese and Teugels, 2005; Eyo and Effiong, 2005; Malla and Ganesh, 2009; Okonkwo and Obiakor, 2010; Awodiran et al., 2015; Hedges et al., 2015; Maneechot et al., 2016).

metaphase chromosomes using fluorescence *in situ* hybridization is required to determine their positions on the sex chromosomes of bighead catfish. A complete high-quality genome assembly for the bighead catfish is required to elucidate the sex-determination mechanism further. This approach will provide basic information with which to explore the sex-determination mechanism and identify potential sex-determination regions in clariid catfish.

Data availability statement

The full data set and metadata from this publication are available from the Dryad Digital Repository Dataset, doi:https://doi.org/10.5061/dryad.prr4xgxj6.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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Genome-Wide SNP Analysis of Hybrid Clariid Fish Reflects the Existence of Polygenic Sex-Determination in the Lineage

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The African catfish (*Clarias gariepinus*) may exhibit the co-existence of XX/XY and ZZ/ZW sex-determination systems (SDSs). However, the SDS of African catfish might be influenced by a polygenic sex-determination (PSD) system, comprising multiple independently segregating sex "switch" loci to determine sex within a species. Here, we aimed to detect the existence of PSD using hybrid. The hybrid produced by crossing male African catfish with female bighead catfish (*C. macrocephalus*, XX/XY) is a good animal model to study SDSs. Determining the SDS of hybrid catfish can help in understanding the interactions between these two complex SDS systems. Using the genotyping-by-sequencing "DART-seq" approach, we detected seven moderately male-linked loci and seventeen female-linked loci across all the examined hybrid specimens. Most of these loci were not sex-linked in the parental species, suggesting that the hybrid exhibits a combination of different alleles. Annotation of the identified sex-linked loci revealed the presence of one female-linked locus homologous with the *B4GALNT1* gene, which is involved in the spermatogenesis pathway and hatchability. However, this locus was not sex-linked in the parental species, and the African catfish might also exhibit PSD.

Keywords: catfish, hybrid, SNP, polygenic sex-determination system, PSD

INTRODUCTION

Sex determination is a basic process in the evolution of sexual reproduction and diverse sex-determination systems (SDSs). Genetic sex determination systems, multifactorial polygenic sex-determining mechanisms, and environmental sex determination systems are found across taxa (Ezaz et al., 2016). Teleosts show a large diversity of SDSs, whereas turnovers or transitions often occur in many vertebrates (Pennell et al., 2018). ZZ/ZW and XX/XY are the common SDSs in the female and male heterogametic systems, and they have evolved repeatedly and independently in the lineage. This

results in the presence of rapidly evolving SDSs in closely related species (Gammerdinger and Kocher, 2018; Nguyen et al., 2021a; Nguyen et al., 2021b). Thus, a study of sister taxa retaining divergent sex chromosome systems would be informative when examining SDS evolution. Among the 115 species of clariid fish (Clariidae), some have either male heterogametic or female heterogametic sex determination systems (Pandey and Lakra, 1997; Nguyen et al., 2021a). Both heterogametic systems are present together, including multiple loci and sex chromosomes in the lineage. Varying SDSs have been observed in African catfish (*Clarias gariepinus*, Burchell, 1822) based on their geographical location: a ZZ/ZW system in Africa, a XX/XY system is indicated in some populations from Israel, Hungary, and China, or both systems in the same population in Thailand (Teugels et al., 1992; Eding et al., 1997; Eyo and Effiong, 2005; Nguyen et al., 2021a). The SDS of African catfish might be influenced by a multifactorial, polygenic sex-determination (PSD) system that occurs in varied species. The occurrence of natural, multiple and independent "switch" loci or alleles specify the segregation of sex within a species. PSD can also occur by modifying current sex chromosomes to generate a third functional sex chromosome at the same locus, or by modifying autosomal loci in other regions of the genome to generate a novel process for gonad development regulation. A well-known example is observed in platyfish (*Xiphophorus maculatus*, Günther, 1866), with some populations showing segregation of male Y chromosome alleles and female W chromosome alleles at the same chromosome pair (Volff and Schartl, 2001). However, the genetic architecture contributing to variations in SDSs is not completely understood, especially in the few known examples of PSD in teleosts (Roberts et al., 2016). Clarification of the genetic structure of SDSs is essential to understand the evolutionary mechanisms and their influence on the processes of speciation (Qvarnström and Bailey, 2009), particularly in species without heteromorphic sex chromosomes.

Interspecific hybridization has successfully improved the growth rate, body size, disease resistance, and tolerance to stressors in aquaculture (Suprapto et al., 2017). For instance, male African catfish (*C. gariepinus*, 2n = 56) have been crossed with female bighead catfish (*Clarias macrocephalus*, Günther, 1864, 2n = 54 with XX/XY) to produce fast growth and improved disease resistance traits in the resulting hybrids (2n = 55) (Na-Nakorn, 1995; Chaivichoo et al., 2020; Nguyen et al., 2021a; Nguyen et al., 2021b). However, reproductive failure can occur as a result of chromosomal incompatibility with parental genomes. This can culminate in spermatogenic breakdown in F_1 male hybrids, leading to subsequent elimination by apoptosis and limiting mass production (Ponjarat et al., 2019). By contrast, F_1 female hybrids can produce copious backcross offspring with low embryo mortality (Na-Nakorn et al., 2004; Abol-Munafi et al., 2006) due to differences in the checkpoint systems in meiotic cells. This may be more stringent during the development of mature spermatozoa than production or development of an ovum or as a result of complex interactions between two different SDSs (Ponjarat et al., 2019). Thus, the male *C. gariepinus* × female *C. macrocephalus* hybrid is a good animal model to study the evolutionary process of SDSs in teleosts. In this study, we proposed the following two hypotheses: 1) the hybrid might possess the dominant SDS from male African catfish when Y or W sex chromosomes is present in different individuals, or 2) the hybrid might exhibit new candidate loci or a combination of different alleles due to the influence of PSD found in male African catfish. To determine how sex determination affects the viability of hybrid catfish, genome-wide single-nucleotide polymorphism (SNP) analyses were performed using Diversity Arrays Technology (DArTseq™) of captive-bred individuals scored with phenotypic sex, as described by Nguyen et al. (2021a) and Nguyen et al. (2021b). Results will provide insights to improve the understanding of SDSs in clariid fish.

MATERIALS AND METHODS

Adult individuals were collected as 15 male and 15 female hybrids, chosen randomly from a breeding stock (Kasetsart University, Bangkok, Thailand) to reduce the possibility of a high occurrence of siblings in the genetic pool. The hybrids were produced from pure breeding stock maintained for 10 years. Six generations of the breeding stock were bred in captivity. The sex of each individual was identified based on external morphology and internal examination of gonadal morphology (Esmaeili et al., 2017; Ponjarat et al., 2019). Animal care and all experimental procedures were approved by the Animal Experiment Committee, Kasetsart University, Thailand (Approval no. ACKU61-SCI-026) and concurred with the Regulations on Animal Experiments at Kasetsart University. The dorsal fins of each individual were removed for DNA extraction and total genomic DNA. DArT sequencing and genotyping, marker selection, DArT sequencing analysis, estimation of expected sex-linked markers, comparison of potential sex-linked loci, and homology searching processes were performed.

Total Genomic DNA

Total genomic DNA was extracted following the salting-out protocol (Supikamolseni et al., 2015). The quality of each extracted DNA specimen was assessed using gel electrophoresis for the presence of high-molecular-weight DNA. All samples were stored at −20°C until required for DArTseq™ library construction (Laopichienpong et al., 2021).

DArT Sequencing and Genotyping

Genotyping of multiple SNP loci was accomplished using Diversity Arrays Technology Pty Ltd. (DArTseq™) in Canberra, ACT, Australia following the methodology of Jaccoud et al. (2001) and Koomgun et al. (2020). The candidate sex-specific loci among male and female individuals were then determined using *in silico* DArT (variability in SNP loci generates presence/absence polymorphism in restriction sites, so-called PA markers). Approximately 100 ng of total DNA from each sample was used to develop the DArTseq™ arrays. The DNA samples were subjected to digestion and ligation reactions, as described by Kilian et al. (2012) and digested using *Pst*I and *Sph*I. The ligation reactions were performed using two adaptors as a *Pst*I compatible adaptor including an Illumina flow-cell

attachment sequence, a sequencing primer and a unique barcode sequence, and a *Sph*I compatible adaptor consisting of an Illumina flow-cell attachment region. The ligated fragments were then processed by 30 cycles of PCR (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 applied to Illumina's proprietary cBot (http://www.illumina.com/products/cbot.html) bridge PCR, followed by sequencing on the Illumina HiSeq 2000 platform. The single read sequencing was run for 77 cycles. Sequences were processed using proprietary DArTseq analytical pipelines (Ren et al., 2015). Poor-quality sequences were filtered by processing the HiSeq 2000 output (FASTQ file). Two quality thresholds were applied to the barcode region for stringent selection as minimum Phred pass score of 30, and a 75% minimum pass length to allow parsing of sequences into specific sample libraries. Relaxed thresholds were applied to the remainder of the sequence as minimum Phred pass score 10 and minimum pass length 50%. Approximately 2,000,000 sequences per barcode/individual were recognized and used in marker calling. Finally, identical sequences were combined into "fastqcoll files" and used in the secondary pipeline (DArTsoft14) for proprietary SNP and PA loci calling, with the "reference-free" algorithm implemented in DArTsoft14. Sequence clusters were then parsed into SNP and *in silico* DArTseq™ markers utilizing a range of metadata parameters derived from the quantity and distribution of each sequence across all analyzed samples. Multiple libraries of the same individual were included in the DArTseq™ genotyping process, enabling reproducibility scores to be calculated for each candidate marker. The outputs generated by DArTsoft14 included reproducibility values at >90%, with read depth >3.5 for SNPs and >5 for PA markers, with call rate >80% (proportion of samples for which the marker was scored) (Koomgun et al., 2020; Laopichienpong et al., 2021; Nguyen et al., 2021a; Nguyen et al., 2021b).

Marker Selection and DArT Sequencing Analysis

Sex-specific loci were derived from the analysis of SNP codominant markers and PA dominant markers. The SNP data were scored for homozygotes to reference allele as "0" (the most common allele), the alternate SNP allele homozygote as "1", the heterozygote as "2", or a score of "–" as the double null/null allele (absence of a fragment remaining the SNP in the genomic representation). The PA data were scored for presence as "1", absence as "0" or a result of "–" for putative heterozygosity. For sex-linked markers in an XX/XY system, reference alleles are often found on the X-chromosome. Here, "SNP alleles" were those that showed polymorphism correlated to the reference allele. SNP alleles from an XX/XY system should be linked with the Y- chromosome and located on or near to the male-determination region if the allele is tightly Y-specific. If the two sex chromosomes recombine in an XX/XY sex chromosome system, SNP alleles should occasionally appear on the X chromosome. If this scenario occurs, then some males might be homozygous for SNP alleles at particular loci. As a result, females could be heterozygous and exhibit a copy of the SNP allele. However, this probability is low. Sex-linked or sex-specific loci were obtained from the analysis of SNP co-dominant markers and PA-dominant markers. For an XX/XY sex chromosome system, the SNP and PA loci sequenced for at least 70%, 80%, 90% and 100% of males were involved in a separate data set. Loci, where all males passed the 100% filtering criterion, were designated as perfectly male-linked, whereas those that passed the 70%–90% criterion were considered moderately male-linked loci (Koomgun et al., 2020; Laopichienpong et al., 2021; Nguyen et al., 2021a; Nguyen et al., 2021b). A similar approach was used to target loci with ZZ/ZW system. The Hamming distance was calculated as the number of pairwise differences between male and female individuals across SNP and PA loci using the "rdist" function in R software (R version 3.5.1). Heatmaps were represented using the function 'pheatmap' in ggplot2 R package (R Core Team, 2021). To evaluate the genetic association between each locus and phenotypic sex, we performed the Cochran-Armitage test (CATT) (Lambert et al., 2016; Sopniewski et al., 2019; Koomgun et al., 2020; Laopichienpong et al., 2021; Panthum et al., 2021). Similarly, the chi-square test was used to assess whether the proportion of different genotypes followed the null expectation. Polymorphism information content (*PIC*) values, as an index for evaluating SNP and PA loci, were calculated for each locus. The *PIC* values ranged from 0 (fixation of one allele) to 0.5 (frequencies of both alleles are equal) (Sven and Klaus, 2019; Koomgun et al., 2020; Laopichienpong et al., 2021; Nguyen et al., 2021a; Nguyen et al., 2021b; R Core Team, 2021).

Estimation of Expected Sex-Linked Markers

The probability of random candidate sex-linked loci showing associations with sex under a small sample size was assessed using the formula $P_i = 0.5^n$, where P is the probability that a given locus, i is sex-linked by chance, 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 (Koomgun et al., 2020; Laopichienpong et al., 2021; Nguyen et al., 2021a; Nguyen et al., 2021b).

Comparison of Potential Sex-Linked Loci

We designated all candidate loci, in which males (in the case of XY) or females (in the case of ZW) were not 100%, to moderately sex-linked loci: 90/10; 80/20; 70/30. Significant differences among the three groups of sex-linked loci were evaluated by the chi-square test using the R package "stats" for PA loci and the Kruskal–Wallis and Nemenyi test using the R package "PMCMR" for SNP loci (R Core Team, 2021), based on the mean heterozygosity and standard deviation for each. All candidate loci were plotted against each individual using the "glPlot" function in the dartR R package (Gruber and Georges, 2021; R Core Team, 2021).

Homology Searches

For all sex-linked loci that met our criteria and had a statistically significant association with phenotypic sex, the hybrid sex-linked loci were searched to find the genome-wide SNP of their parents (*Clarias gariepinus* × *Clarias macrocephalus*) and detect the inherited loci. In this study, genome-wide SNP of *Clarias gariepinus* × *Clarias*

TABLE 1 | DArT analysis for 15 male and 15 female individuals of hybrid catfish (*Clarias gariepinus*, Burchell, 1822 x *Clarias macrocephalus*, Günther, 1864).

ZZ/ZW sex-determination type	**30:70 male:female**		**20:80 male:female**		**10:90 male: female**		**0:100 male: female**	
	SNP[a]	**PA[b]**	**SNP[a]**	**PA[b]**	**SNP[a]**	**PA[b]**	**SNP[a]**	**PA[b]**
Total number of DArT analyses	50,348	51,294	50,348	51,294				
Sex-linked loci	7	128	1	16	–	–	–	–
Overall mean distance between males and females	0.669 ± 0.019	0.712 ± 0.008	0.760 ± 0.029	0.745 ± 0.009	–	–	–	–
Overall mean distance within females	0.382 ± 0.026	0.397 ± 0.019	0.343 ± 0.047	0.338 ± 0.018	–	–	–	–
Overall mean distance within males	0.370 ± 0.032	0.613 ± 0.011	0.133 ± 0.033	0.603 ± 0.017	–	–	–	–
CATT test	χ^2 = 5.231–16.425 $p < .022$	χ^2 = 4.320–13.715 $p < .037$	χ^2 = 16.425 $p < .000$	χ^2 = 6.250–13.715 $p < .012$	–	–	–	–
XX/XY sex-determination type	**70:30 male:female**		**80:20 male:female**		**90:10 male: female**		**100:0 male: female**	
	SNP[a]	**PA[b]**	**SNP[a]**	**PA[b]**	**SNP[a]**	**PA[b]**	**SNP[a]**	**PA[b]**
Sex-linked loci	7	97	–	7	–	–	–	–
Overall mean distance between males and females	0.680 ± 0.014	0.719 ± 0.018	–	0.778 ± 0.020	–	–	–	–
Overall mean distance within females	0.457 ± 0.021	0.517 ± 0.033	–	0.539 ± 0.038	–	–	–	–
Overall mean distance within males	0.367 ± 0.02	0.403 ± 0.022	–	0.318 ± 0.022	–	–	–	–
CATT test	χ^2 = 6.533–10.995 $p < .001$	χ^2 = 4.572–16.250 $p < .032$	–	χ^2 = 9.458–16.250 $p < .002$	–	–	–	–

[a]*SNP, Single-nucleotide polymorphic loci.*
[b]*PA, Restriction fragment presence/absence loci.*

macrocephalus was used from our previous study as described by Nguyen et al. (2021a); Nguyen et al. (2021b). NCBI BLAST was performed to discover homologies of sex-linked SNP/PA loci against a selection of available teleost fish genomes including Japanese rice fish (*Oryzias latipes*, Temminck and Schlegel, 1846), zebrafish (*Danio rerio*, Hamilton, 1822), Japanese pufferfish (*Takifugu rubripes*, Temminck and Schlegel, 1850), channel catfish (*Ictalurus punctatus*, Rafinesque, 1818), and chicken (*Gallus*, Linnaeus, 1758) (**Supplementary Table S1**). These species were selected as representative of reference genomes due to the availability of high-quality gene annotations and almost complete up-to-date assemblies (Takeda, 2008; Warren et al., 2017). A BLAST homology search was performed in two rounds. First, we aligned sex-linked loci against the reference teleost genome, and then mapped the homologous genes to further clarify their location on sex chromosomes of high-quality annotated genomes that were representative of vertebrate species. Sex-linked loci were used to search against both the NCBI "nr" (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the RepBase version 19.11 database (Genetic Information Research Institute; https://www.girinst.org/) (Bao et al., 2015). RepBase is a database of transposable elements including DNA transposons, LTR and non-LTR *retrotransposons* and endogenous retroviruses (ERVs). We chose criteria of E-values lower than .005 and query coverage with similarity of more than 60%.

RESULTS

The DArTseq methodology identified 50,348 SNP loci and 51,294 PA loci. Polymorphism information content values ranged from 0 to 0.50 for all loci, suggesting that the overall distribution of *PIC* values was asymmetrical and skewed toward higher values. Several SNP and PA loci were selected with a varying set of criteria (**Table 1**; **Figure 1** and **Figure 2**) and compared to determine the existence of XX/XY or ZZ/ZW SDS in the hybrid. In the case of ZZ/ZW type, filtering using the criterion of 30:70 male:female showed in seven SNP loci and 128 PA loci as moderately female-linked (**Supplementary Table S2**, **Figure 2**). Hamming distances between male and female individuals using the moderately sex-linked SNP and PA loci indicated within-sex distances of 0.370 ± 0.032 in males and 0.382 ± 0.026 in females for SNP loci, and 0.613 ± 0.011 in males and 0.397 ± 0.019 in females for PA loci. Between-sex distances were 0.669 ± 0.019 for SNP loci and 0.712 ± 0.008 for PA loci. The CATT results showed a statistically significant association with the phenotypic sex for seven SNP loci (χ^2 = 5.231–16.425, $p < .022$) and 128 PA loci (χ^2 = 4.320–13.715, $p < .037$) (**Table 1**; **Supplementary Table S2**, **Figure 1**). The criterion of 20:80 male:female involved the designation of one SNP locus and sixteen PA loci as moderately female-linked (**Supplementary Table S2**, **Figure 2**). Proportional pairwise Hamming distances between males and females using the moderately sex-linked SNP and PA loci (under the null exclusive model) showed within-sex distances of 0.133 ± 0.033 in males and 0.343 ± 0.047 in females for SNP loci, and 0.603 ± 0.017 in males and 0.338 ± 0.018 in females for PA loci. Between-sex distances were 0.760 ± 0.029 for SNP loci and 0.745 ± 0.009 for PA loci. The CATT results verified a significant association with phenotypic sex for one SNP locus

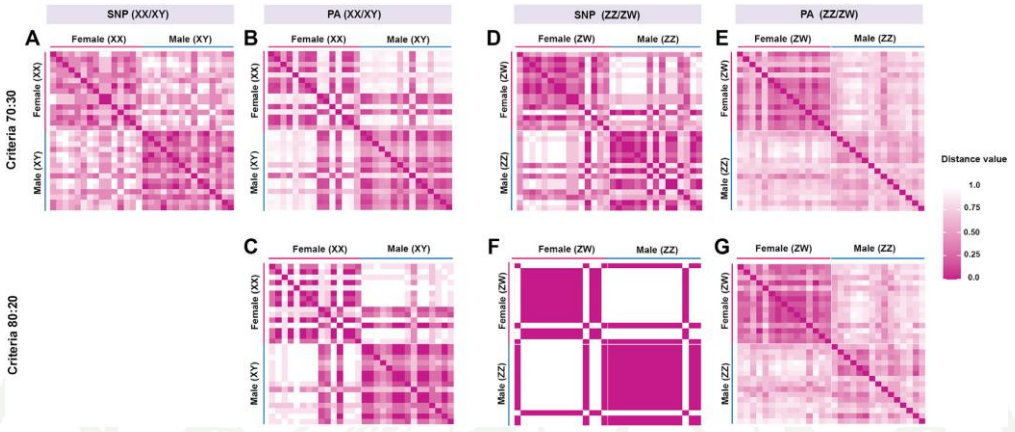

FIGURE 1 | Hamming distances between male and female individuals of hybrid catfish (*Clarias gariepinus*, Burchell, 1822 x *Clarias macrocephalus*, Günther, 1864). The loci were filtered under different criteria. **(A)** Single-nucleotide polymorphic (SNP) loci filtered under the criterion 70:30 (male:female), **(B)** Restriction fragment presence/absence (PA) loci under the criterion 70:30 (male:female), **(C)** PA loci under the criterion 80:20 (male:female), **(D)** SNP loci under the criterion 30:70 (male: female), **(E)** PA loci under the criterion 30:70 (male:female), **(F)** SNP loci under the criterion 20:80 (male:female) and **(G)** PA loci under the criterion 20:80 (male: female).

(χ^2 = 16.425, p < 0.000) and 16 PA loci (χ^2 = 6.250–13.715, p < 0.012) (**Table 1**; **Supplementary Table S2**, **Figure 1**).

For the XX/XY determination system, filtering using the criterion of 70:30 male:female yielded seven SNP loci and 97 PA loci that were male-linked (**Supplementary Table S3**, **Figure 2**). Hamming distances between male and female bighead catfish using sex-linked SNP and PA loci (under the null exclusive model) showed lower within-sex distances of 0.367 ± 0.02 in males and 0.457 ± 0.021 in females for SNP loci, and 0.403 ± 0.022 in males and 0.517 ± 0.033 in females for PA loci. Between-sex distances of 0.680 ± 0.014 for SNP loci and 0.719 ± 0.018 for PA loci were observed. CATT results verified the significance of associations, with phenotypic sex for seven SNP loci (χ^2 = 6.533–10.995, p < .001) and 97 PA loci (χ^2 = 4.572–16.250, p < .032) (**Table 1**; **Supplementary Table S3**, **Figure 1**). No SNP loci and seven PA loci were associated with males based on the criterion of 80:20 male:female (**Supplementary Table S3**, **Figure 2**). Hamming distances between male and female hybrid catfish using sex-linked PA loci showed lower within-sex distances 0.318 ± 0.022 in males and 0.539 ± 0.038 in females for PA loci. Between-sex distances of 0.778 ± 0.020 for PA loci were observed. CATT results verified significant associations with phenotypic sex for seven PA loci (χ^2 = 9.458–16.250, p < .002) (**Table 1**; **Supplementary Table S3**, **Figure 1**). A glPlot showed similarity between sexes in the sample group for both XX/XY and ZZ/ZW SDSs when considering moderately sex-linked loci (**Figure 3**). Female sex-linked loci and male sex-linked loci of hybrid catfish indicated sequence homology with vertebrate genomes, based on global BLAST analyses of NCBI databases (**Supplementary Tables S2,3**). Regarding the ZZ/ZW type, ten of the 135 SNP and PA loci were homologous to the putative genes. Moreover, two SNP loci and 18 PA loci demonstrated partial homology with transposable elements (TEs) (**Supplementary Tables S4,5**). For PA loci, chi-square tests showed that the 30:70 and 20:80 filtering criteria demonstrated insignificant differences in males (χ^2 = 5.388 × 10^{-32}, p = 1) and females (χ^2 = 1.959 × 10^{-33}, p = 1). For SNP loci, Kruskal–Wallis tests indicated that these filtering criteria produced insignificantly different percentages of heterozygosity in males (H = 2.860, p = .091) and females (H = 6.840, p = .009) (**Supplementary Figure S1**). Calculation of pairwise comparisons using the Nemenyi-tests with chi-square approximation for independent samples revealed that the 30:70 and 20:80 filtering criteria resulted in insignificant differences in heterozygosity compared with other filters for males (p = .143) and females (p = .571). By contrast, with regard to the XX/XY sex chromosome system, six of 104 SNP and PA loci were homologous to the putative genes. Two SNP loci and twenty-one PA loci showed partial homology with TEs (**Supplementary Tables S6,7**). Chi-square tests showed that the 70:30 and 80:20 filtering criteria indicated insignificant differences in males (χ^2 = 3.962 × 10^{-32}, p = 1) and females (χ^2 = 2.518 × 10^{-32}, p = 1) for PA loci. The hybrid sex-linked loci were searched to find the genome-wide SNP of their parents (*C. gariepinus* × *C. macrocephalus*) and detect the loci inherited from their parents. The genome-wide SNP of their parents (*C. gariepinus* × *C. macrocephalus*) were used from our previous study, as described by Nguyen et al. (2021a); Nguyen et al. (2021b). Twenty-six candidate

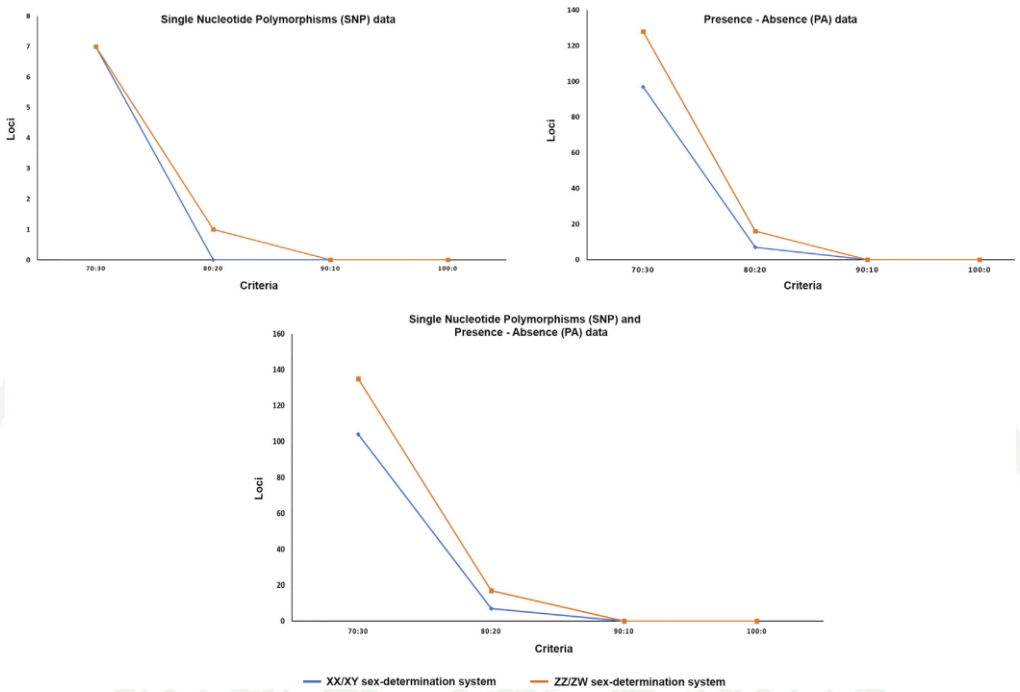

FIGURE 2 | Graph showing number of loci for different hypotheses of sex determination systems after filtering with different criteria. *X*-axis indicates the number of loci and *Y*-axis shows the filter criteria.

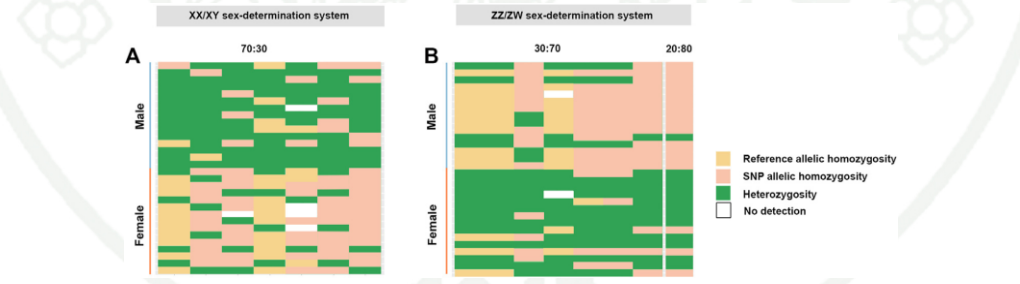

FIGURE 3 | (A) Index of seven sex-linked loci filtered under the criterion 70:30 (male:female) (XX/XY sex-determination system) and **(B)** index of seven sex-linked loci filtered under the criteria 30:70 and index of one sex-linked loci filtered under the criteria 20:80 (male:female) (ZZ/ZW sex-determination system). Plots were generated using the "gIPoIt" function in the R package dartR (Gruber and Georges, 2021). Yellow shading indicates reference allelic homozygosity, green is indicative of heterozygosity and pink indicates SNP allelic homozygosity.

sex-linked markers were found in bighead catfish and three candidate sex-linked markers were found in African catfish, while 75 of the total 84 candidate sex-linked loci of the bighead catfish were found in the hybrids (**Supplementary Table S8**).

Across a range of sample sizes and loci, 30 phenotypically sexed individuals are essential to minimize the probability of selecting less than one spurious sex-linked marker. The probability (P_i) of a single locus exhibited a sex-linked pattern by chance was 9.31×10^{-10}. For the full data set (no filtering), out

of 101,642 loci (including SNP and PA loci), 9.463×10^{-5} were expected to spuriously show perfect sex-linked loci based on chance alone.

DISCUSSION

Although the SDS of the last common ancestor of clariid fishes is not known, the hybrid catfish in this study provided a unique opportunity to study the various SDSs that are present in clariid fishes. Most SDSs are XY or ZW homomorphic sex chromosomes (Nguyen et al., 2021a). Due to the lability of sex determination processes in teleosts, closely related species sometimes possess different SDSs (Kitano and Peichel, 2012). Both sex determination systems might have undergone collateral evolution, or a transformation might have occurred in the common ancestor, which separated the lineages. Here, the genome-wide SNP of their parent (*C. gariepinus* x *C. macrocephalus*) was used from our previous research, as described by Nguyen et al. (2021a); Nguyen et al. (2021b). Female bighead catfish can produce only X gametes, whereas male African catfish can produce possible X, Y, and Z gametes. If both systems are present in the African catfish population in Thailand, their sex-linked SNP loci can be independently transferred from the male parent to the hybrid, thereby resulting in the presence of different SNP profiles in each individual. In this study, 26 candidate sex-linked markers came from the bighead catfish and three candidate sex-linked markers came from the African catfish. Seventy-five of the total 84 candidate sex-linked loci of the bighead catfish were observed in the hybrids but they were not all sex-linked loci in the hybrid (**Supplementary Tables S8**). This suggests that several sex-determining loci or clusters remain in the parental African catfish. Developmental pathways that are downstream of the primary sex-determining genes are conserved among various organisms having vastly different SDSs (Ezaz et al., 2016). These results collectively suggest that the variation in the SDSs of the parents might result in PSD in the hybrid. Male F_1 hybrids are sterile because of the influence of different numbers of diploid chromosomes in their parents or because of the presence of complex SDSs. Sex-linked genes are important to determine the incompatibility between hybrids and the presence of non-homologous sex chromosomes could indicate reproductive isolation and increase the probability of speciation (Qvarnström and Bailey, 2009). In our previous study, we have shown that all sex-linked loci in African catfish and bighead catfish were not found in the same linkage group (Nguyen et al., 2021a; Nguyen et al., 2021b). Therefore, we predict that African catfish exhibit PSD on different chromosomes. PSD results in the generation of multiple phenotypic or reproductive classes within one sex. In the African catfish, more than two sex types have been observed in diverse populations (Teugels et al., 1992; Eyo and Effiong, 2005; Nguyen et al., 2021a). The presence of multiple classes may promote fitness benefits as natural selection in the population, suggesting that PSD engenders an evolutionarily stable scenario. Moreover, there is a noticeable diversity in how PSD has evolved separately across teleosts (Sandra and Norma, 2010). Genetic conflicts may also markedly impact hybrid sex determination and gametogenesis, with outcomes as intersex or sterile individuals, respectively and this can build strong post-zygotic barriers. PSD systems are evolutionarily unstable, because of sex-specific natural selection; they have generally been evaluated as only a midway step in the evolution of SDSs (Bachtrog et al., 2014).

Several reasons for the emergence of differences in SDSs in two closely related species have been postulated. With no sex chromosomes in the last common ancestor, both systems developed independently or one lineage retained the ancestral sex chromosomes, while transition occurred in other lineages (Mank et al., 2006). Such transitions can occur on the same pair of sex chromosomes or involved as an autosome that then became a new sex chromosome (Franchini et al., 2018). Sex-determining gene(s) at the molecular level may change their mode of action from female to male determination. Alternatively, a novel sex-determining (SD) gene might have initiated SD turnover in the evolving lineage. This change in mode of action of the genes may occur in the lineage of clariid fish, where both XX/XY and ZZ/ZW systems are found (Nguyen et al., 2021a; Nguyen et al., 2021b). By contrast, the platyfish has XY system, which is an ancestral SDS. One X chromosome acquired a female sex-determination allele dominant to the Y chromosome. This resulted in WY individuals developing ovaries. Several species of cichlid fish from Lake Malawi exhibited both an XY locus and WZ locus on different chromosome pairs. With occurrence on the same individual, the W female determines the dominant locus and the selected individual consequently develops as female (Ser et al., 2010). Ultimately, the dominant locus determines the fate of the gonad in the system being examined. Modification of existing sex chromosomes leads to multifactorial mechanisms, creating a functional third sex chromosome at the same or different locus. In this study, the female hybrids were fertile and able to produce large numbers of backcross progeny (Na-Nakorn et al., 2004; Ezaz et al., 2016). This suggested that the systems remain partially compatible (if one supersedes the other). We determined seven SNP or PA markers for male-linked loci and 17 SNP or PA markers for female-linked loci, with the criterion of 80:20 male: female across all examined hybrid specimens. Comparison of sex-linked loci of hybrids and the parent species showed no tendency of inheritance. Many false-positive signals might be desirable from such specimens due to their diverse genetic backgrounds (Gamble et al., 2015). Current data exhibited the possibility of this approach. The probability that a single locus exhibited sex linkage pattern by chance was 9.31×10^{-10}, and when the full data set (without filtering) of 101,642 loci (including SNP and PA loci) was considered, 9.463×10^{-5} were expected to spuriously indicate sex-linkage. Thus, identification of any erroneously sex-linked loci by chance seems unlikely. This suggests the possibility of the presence of novel sex-linked genetic loci as a consequence of combination polygenes. Combining independently evolved sex chromosomes could also stimulate new combinations of sexual characters (Tanaka et al., 2007). Sex determination in zebrafish and cichlids likely results from a combination of additive and epistatic interactions at many loci (Parnell and Streelman, 2013). Surprisingly, the PA35637844 female-linked locus of the hybrid

in the present study showed homology with the *B4GALNT1* gene, and this locus was derived from a non-sex-linked locus found in the bighead catfish. Our gene ontology search in the Ensembl v103 database confirmed that the *B4GALNT1* gene encodes functions related to the spermatogenesis pathway and hatchability (Izumi et al., 2019). Hence, the PA35637844 locus might have a putative role in the sex determination of the hybrids. An allele that wins in one combination of sex determination loci genotypes may lose in another, as observed from sex-linked loci of the bighead catfish. However, the influence of PSD remains in hybrids and African catfish. These outcomes illustrate the difficulty in predicting the consequences of SDSs on the speciation process that has only been studied in some cases.

CONCLUSION

Hybrid catfish is of considerable economic importance for aquaculture as they can improve productivity through hybrid vigor, produce sterile animals or transfer desirable traits. Our findings revealed that SDS type ZZ/ZW can co-exist with XX/XY types as PSD in the same individuals of hybrid catfish. These models can be used to study the evolution of gene networks and epistasis, thereby allowing us to investigate the developmental regulation of genes that are traditionally thought to be members of core sex signaling networks. Identifying additional sex-determining genes and their interactions can offer unique insight to infer the process of sexual development. However, our results need to be extrapolated to other catfish species to investigate the evolution of SDSs in clariid fish. Chromosome mapping using fluorescence *in situ* hybridization on sex-linked loci and high-quality whole genome assemblies should be performed in catfish to identify the positions of sex-determining genes/loci.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://datadryad.org/stash, 10.5061/dryad.prr4xgxj6.

ETHICS STATEMENT

The animal study and experimental procedures were reviewed and approved by the Animal Experiment Committee, Kasetsart University, Thailand (Approval no. ACKU61-SCI-026) and conducted in accordance with the Regulations on Animal Experiments at Kasetsart University. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

DHMN and KS were the major contributors in writing the manuscript; DHMN, EK, and KS formulated the ideas and designed the methodology; DHMN and KS detailed the experiments; DHMN, EK, and KS performed the data analysis; DHMN, JP, NL, TP, WS, SA, EK, NM, PD, SP, UN, and KS reviewed the data and the manuscript. All authors have read and approved the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2022.789573/full#supplementary-material

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Conclusions

Hybrid catfish play an important role in Thailand aquaculture. However, hybrids are sterile due to variable reproductive failure, limiting mass production of the F_1 hybrid. Therefore, genetic improvement in the breeding management of North African catfish, bighead catfish and their hybrids is critical.

This research presented a number of moderately sex-linked loci using a genome-wide SNP approach. Results indicated that the male heterogametic XX/XY sex-determination system should co-exist with the ZW system in the same individuals of *C. gariepinus*. The SDS of African catfish might be influenced by a polygenic sex-determination (PSD) system but the locations of genomic regions associated with sex chromosome differentiation remain unclear. Male-linked loci on the putative Y sex chromosome of the bighead catfish occupy a small portion of the genome. Results suggest the presence of XX/XY sex determination in bighead catfish. One male-linked locus exhibited homology with the *GTSF1L* gene, demonstrating an expression pattern enriched in testes, while another showed partial homology with the Z sex chromosomes of the western terrestrial garter snake and chicken. Moreover, a PCR-based DNA marker validated the male-linked loci in bighead catfish. Results showed that ZZ/ZW SDS can co-exist with XX/XY SDS as PSD in the same individuals of hybrid catfish. Seven moderately male-linked loci and seventeen female-linked loci were collected across all the hybrid individuals. Most of these loci were not sex-linked in the parental species. Twenty-six candidate sex-linked markers were sourced from bighead catfish, while three candidate sex-linked markers came from African catfish. Seventy-five of the total 84 candidate sex-linked loci of bighead catfish were observed in the hybrids but were not all sex-linked loci. This suggests that several sex-determining loci remain in the parental African catfish and that variation in the SDSs of the parents might result in PSD in the hybrid. One female-linked locus was homologous with the *B4GALNT1* gene, which is involved in the spermatogenesis pathway and hatchability. This approach provides basic information to study the sex-determination mechanisms and identify potential sex-determination regions in clariid catfish.

Recommendations for future work

The relevance of sex-determination systems in understanding the evolutionary and speciation processes and the genetic architecture of Clariid catfish remains unknown. Therefore, a complete high-quality genome assembly for the African catfish, bighead catfish and their hybrids is essential to elucidate the sex-determination mechanism and develop genotypic sex assays. This research will provide a baseline for genetic manipulation and breeding programs.

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