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Characterization of the sex determining region of channel catfish (*Ictalurus punctatus*) and development of a sex-genotyping test

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ABSTRACT

Channel catfish is an important species for aquaculture that exhibits a sexually dimorphic growth in favor of males. Genetic sexing and development of sex markers are crucial for the early identification of sex and of particular genotypes (YY males) for the production of all-male population in channel catfish aquaculture. In this study, we sequenced genomic DNA from pools of males and pools of females to better characterize the sex determining region (SDR) of channel catfish and to develop sex-specific markers for genetic sexing. Performing comparative analyses on male and female pooled genomic reads, we identified a large SDR (~8.3 Mb) in the middle of channel catfish linkage group 4 (LG04). This non-recombining SDR contains a high-density of male-specific (Y chromosome) fixed single nucleotide polymorphisms (SNPs) along with ~ 185 kb male-specific insertions or deletions. This SDR contains 95 annotated protein-encoding genes, including the recently reported putative channel catfish master sex determining (MSD) gene, breast cancer anti-estrogen resistance protein 1 (*bcar1*), located at one edge of the SDR. No sex-specific SNPs and/or indels were found in the coding sequence of *bcar1*, but one male-specific SNP was identified in its first intron. Based on this genomic information, we developed a PCR-based sex-specific genetic test. Genotyping results confirmed strong linkage between phenotypic sexes and the identified SDR in channel catfish. Our results confirm, using a Pool-Seq approach, that channel catfish is male heterogametic (XX-XY) with a large SDR on the LG04 sex chromosome. Furthermore, our genotyping primers can be used to identify XX, XY, and YY fish that will facilitate future research on sex determination and aquaculture applications in channel catfish.

1. Introduction

Channel catfish, *Ictalurus punctatus*, belongs to the Ictaluridae family within the order Siluriformes (catfishes), and is known as a highly adaptive species with a high tolerance to low water quality and resistance to various infectious pathogens (Liu et al., 2016). Its native geographic distribution encompasses the south-east of Canada, the eastern part of the United States, and northern Mexico. It is now widely farmed worldwide and is an important aquaculture species especially in North America and in Asia where it has been introduced. Channel catfish exhibits a sexual growth dimorphism with male growing faster than female (Simco et al., 1989). Therefore, the establishment of all-male mono-sex populations of channel catfish is sought after for aquaculture production (Simco et al., 1989). This made research on sex determination (SD) mechanisms in channel catfish especially interesting. Channel catfish SD system has been described as being male heterogametic (XX/XY) with morphologically undifferentiated sex chromosomes (Tiersch et al., 1990). This study identified the breast cancer anti-

Abbreviations: SD, sex determination; SDR, sex differentiated regions; MSD, Master sex determining; SNPs, single nucleotide polymorphisms; LG, linkage group; GSD, genetic sex determination; GSD+TE, thermal effects on GSD; Pool-seq, Whole genome pool-sequencing.

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estrogen resistance protein 1 gene (*bcar1*) as the potential channel catfish master sex determining (MSD) gene encoding a male-specific isoform transcript (Bao et al., 2019).

In the teleost fish, SD mechanisms are highly diverse with either genetic or environmental (temperature, pH, social interactions) triggers (Baroiller and D'cotta, 2001; Kobayashi et al., 2013). In channel catfish, like in some other aquaculture fish species such as goldfish and Nile tilapia, previous studies also reported that SD could be under genetic determinants (genetic sex determination, GSD) with an influence of temperature (Patiño et al., 1996; Goto-Kazeto et al., 2006; Wen et al., 1758; Wessels and Hörstgen-Schwark, 2011; Hattori et al., 2007), a phenomenon known as thermal effects on GSD (GSD + TE) (Ospina-Alvarez and Piferrer, 2008). However, in channel catfish the effective temperature threshold for triggering male-to-female sex-reversal is 34 °C (Patiño et al., 1996); questioning the significance of this GSD + TE effect in wildtype populations of channel catfish. GSD in teleost is characterized by a high turnover of SD systems, sex chromosomes, and MSD genes. Classical mono-factorial male (XX/XY) or female (ZZ/ZW) heterogametic SD systems have been described (Devlin and Nagahama, 2002) along with multiple sex chromosomes (Sember et al., 1833; Moore and Roberts, 2013). This high SD turnover (Pan et al., 2016) is even observed among closely-related species (Matsuda and Sakaizumi, 2016) or different populations of the same species (Dong et al., 2021; Pan et al., 2021). This plasticity in SD of teleost makes them interesting models to study the evolution of SD in vertebrates.

Growth is one of the most valuable economic traits in fish and benefiting from a better growth rate of either males or females is key for modern aquaculture practices (Mei and Gui, 2015). In most teleost fish, sex chromosomes are cytogenetically and morphologically undifferentiated (Sandra and Norma, 2009), but it is feasible to develop molecular approaches to identify sex markers in fish for aquaculture applications. These molecular approaches firstly used different molecular marker techniques such as amplified fragment length polymorphism (AFLP) (Felip et al., 2005; Lee et al., 2011), random amplified polymorphism DNA marker technology (random amplified polymorphic DNA, RAPD) (Vale et al., 2014; Kovács and Egedi, 2000), microsatellite marker technology simple sequence repeats (SSR) (Shikano, 2011; Fuji et al., 2010). With the development of high throughput sequencing technologies, sex-linked markers were successfully characterized using Restriction-site Associated DNA (RAD) sequencing (Kafkas et al., 2015; Gamble, 2016), Quantitative Trait Loci (QTL) mapping (Dong et al., 2019; Zhou et al., 2021), and whole-genome sequencing (Wen et al., 2022; Feron et al., 2020). In the present study, we implemented males versus females pooled whole genome sequencing approach (Pool-sequencing, Pool-Seq) (Futschik and Schlötterer, 2010) to identify sex-linked genetic variations in channel catfish, leveraging the available genome assemblies of an XX female (Liu et al., 2016) and a YY male (Bao et al., 2019). With this strategy, we identified a large SDR on channel catfish linkage group 4 (LG04), confirming previous results demonstrating the LG04 was the sex chromosome in that species (Bao et al., 2019). We also developed sex-specific markers for channel catfish that will be important for SD research and the development of genetically all-male population for channel catfish aquaculture.

2. Materials and methods

2.1. Fish sampling and genomic DNA extraction

Fish used in this experiment were collected from a wild population in the Wisconsin River (Grant county, Wisconsin state, USA) and a cultured population in South Central Freshwater Fish R&D Center of Charoen Pokphand Group, China. The phenotypic sex of the 49 males and 35 females sampled was identified based on sex-specific differences in the vent area of each fish. Fin clips were stored in 95 % ethanol until genomic DNA (gDNA) extraction.

For routine sex genotyping, fin clips were cut into pieces, and lysed by 20 mg Proteinase K within 5 % Chelex at 55 °C for 2 h, followed by Proteinase K denatured at 99 °C for 2 min. After brief centrifugation, the supernatant containing genomic DNA was transferred to a new PCR tube. Finally, DNA was diluted with an equal volume of water and stored at −20 °C before genotyping.

For Pool-Seq, Fin clips' gDNA from 30 phenotypic females and 30 males were extracted individually using the NucleoSpin Kits for Tissue (Macherey-Nagel, Duren, Germany) following the manufacturer's instructions. Concentrations of gDNA were measured using a Qubit3 flurometer and the Qubit dsDNA HS Assay Kit (Invitrogen, Carlsbad, CA), and gDNA from each individual was normalized to reach equimolar gDNA concentration within each sex. All normalized gDNA were then pooled according to sex to produce equimolar gDNA pools of males and females.

2.2. Whole genome pool-sequencing (Pool-seq)

Pool-seq libraries (male and female pools) were constructed using the Truseq nano kit (Illumina, ref. FC-121-4001) following the manufacturer's instructions. Briefly, each pool was sonicated using a Bioruptor (Diagenode). Firstly, 200 ng of gDNA from the male and female pools was briefly sonicated with Bioruptor sonication device (Diagenode, Liege, Belgium). The sonicated DNA was size selected on magnetic beads filtering for a 550 bp insert size and adenylated on their 3′ ends. Adenylated DNA was ligated to Illumina's specific adapters and, after purification on magnetic beads, was amplified in an 8 cycles PCR. Libraries were purified using magnetic beads, checked on a Fragment Analyzer (Agilent) using the HS NGS Fragment kit (DNF-474-33), and quantified by qPCR using the KAPA Library quantification kit (Roche, ref. KK4824). Finally, 2 x150 paired reads for the male and female pools were generated.

2.3. Identification of sex determining region (SDR)

The published two genome assemblies, one XX female [GCA_001660625.2] and one YY male [GCA_004006655.3] were used as genome references for our whole genome analysis. Illumina reads, from both male and female pools, were respectively mapped onto the two individual assemblies using BWA mem version 0.7.17 (Li and Durbin, 2009) with the default setting. Then, for each assembly, the aligned sequences were sorted, grouped, and merged using the default settings of Picard tool version 2.18.2 (https://broadinstitute.github.io/picard/). PCR duplicates were removed using the Picard tool. In addition, reads with mapping quality less than 20 and that not mapped uniquely were removed using *samtools* version 1.9 (Danecek et al., 2021). *Samtools mpileup* was then used to generate a *pileup* file using the two sex BAM files by setting per-base alignment quality disabled (-B). Subsequently, with this mpileup file a sync file was generated using popoolation *mpileup2sync* version 1.201 (Kofler et al., 2011) by setting the minimum quality to 20. The nucleotide composition of each sex for each position in the reference was included in the sync file. SNPs and window coverages of the male and female pooled datasets were calculated using PSASS (https://github.com/SexGenomicsToolkit/PSASS). To identify sex-biased SNP enriched region, a 100 kb sliding window with an output every 10 kb was applied using PSASS. The parameters for running PSASS were as follows: minimum depth to consider a site was set to 10 (–min-depth 10), range of frequency for a sex-linked SNP in the heterogametic sex was set to 0.5 ± 0.2 (–freq-het 0.5, –range-het 0.1), frequency of a sex-linked SNP in the homogametic sex set to more than 0.95 (–freq-hom 1, –range-hom 0.02). The resulting analyses were plotted the PSASS supporting R package (https://github.com/RomainFeron/PSASS-vis).

2.4. Sex-specific primers design, SNP verification, and genotyping

The channel catfish SDR identified with the Pool-Seq approach described above was visually inspected with the Integrative Genomics

Viewer (IGV) (Thorvaldsdottir et al., 2013) to find short insertions or deletions characterized by a half read coverage in one sex and no or very few reads (less than 2X) in the other sex. Primers were designed flanking these potential sex-specific fragments using Primer3 version 4.1.0 (https://primer3.ut.ee). The same strategy was applied to design two primer sets amplifying fragments containing sex-specific SNPs. All primers are listed in Table S1 and PCRs were performed with 0.25 units of JumpStart Taq DNA Polymerase (Sigma Aldrich), 1 μl of 10 × PCR Buffer, 100 μM dNTP mixture, 1 μl of 50 ng/μl gDNA, 0.1 μM of each primer in a total volume of 25 μl. The conditions of PCR were: denaturation at 94 °C for 30 s, primer annealing at 56 °C for 30 s, and polymerase extension at 72 °C for 30 s for 35 cycles. Finally, PCR products were visualized on 1.5 % agarose gels.

3. Results

3.1. Whole genome pool-sequencing data of channel catfish

Whole genome sequencing of male and female pools yielded respectively 266,697,484 and 231,722,384 paired-end clean reads in total (Table 1). With the help of genome reference of YY channel fish, mapping rates of paired-end reads from male pool and female pool were 99.55 % and 99.50 % respectively, and the average depth of the male pool and female pool were 34 and 30 respectively. Besides, the nucleotide statistics of sequencing data showed that the GC contents were 40.0 % and 40.0 %, the averages of Q20 were 96.7 % and 96.8 %, and the averages of Q30 were 91.9 and 92.0 in the male pool and female pool respectively (Table 1). These results show that the sequencing datasets of the male and female channel catfish pools are of high and equivalent quality.

3.2. Identification of sex chromosome and sex-differentiation region in channel catfish

To characterize the sex-chromosome and SDR of channel catfish, we first searched for sex-biased signals, including sex coverage differences and sex-biased SNPs using the published channel catfish genome as a reference. The whole genome analysis of the sex-specific SNP distribution using the male assembly as a reference showed that a high density of male-biased SNPs was present on LG04 (Y chromosome) (Fig. 1, Fig. 2B, and 2C). A similar SDR region was also identified on LG04 (X chromosome) using the female assembly as a reference (Figure S1, Fig. S2B). Furthermore, population genetics analyses showed significant genetic differentiation with a high F_{ST} value, between males and females, were observed in the position of SDR (Fig. 2A, Fig. S2A). These results confirmed previously published results describing LG04 as the sex-chromosome in channel catfish (Liu et al., 2016), but differences in the distribution of male-biased SNPs were observed in our Pool-Seq analyses depending on the reference assembly used. With the male genome assembly as a reference, male-biased SNPs were more tightly clustered (Fig. 2B, Fig. S2B) in an 8.3 Mb SDR compared to a 9.8 Mb SDR with the female assembly. Comparison between X and Y chromosomes showed a good alignment in the pseudo-autosomal regions around the SDR (Figure S3), whereas, the X and Y alignments were fragmented in the SDR (Figure S3), giving rise to the discrepancy in the SNP distribution pattern observed in our data. Furthermore, these small structural changes between the X and Y chromosome could potentially be involved in the suppression of the recombination between sex chromosomes.

3.3. Development of sex-specific chromosome markers and SNPs verification in channel catfish

Using Integrative Genomics Viewer (IGV), we manually checked the mapping data throughout the Y chromosome and identified around 185 kb of potential Y-specific insertions based on coverage difference between the male and female pools (Supplemental excel file 3). Then we designed three PCR primer sets in conserved male and female (Y and X) regions around some of this small male-specific (Y-specific) insertions (Figure S4). These three sex-linked primer sets produce PCR products of different sizes in males and females (Fig. 3A) with a single amplicon in females (X-amplicon) and two amplicons in males (X- and Y- amplicons) (Fig. 3B). PCR of 12 additional phenotypic males and 26 phenotypic females of the wild population (new individuals not included in the male and female pools) demonstrated a complete association (p-value = 7.08e-09) of the phenotypic sex with the sex genotype (XX or XY) inferred from the PCR tests for all three sex-specific primer sets used (Figure S5). And these sex-specific primers were also verified in the cultured population with 12 males and 16 females (Figure S7). In addition, we also designed conserved X and Y primers around male-specific SNPs and four SNPs with specific heterozygosity in males were verified by Sanger sequencing of the resulting PCR products sequencing (Fig. 3C).

3.4. Search for potential MSD genes in the channel catfish SD

The MSD gene controlling genetic SD in channel catfish likely resides in the SDR on the Y chromosome (Zhang et al., 2022). We extracted all protein-encoding genes (N = 95) from the 8.3 Mb SDR on the Y chromosome that we characterized using Pool-Seq (Supplemental excel file 1). In the Y SDR, we observed 10,190 male SNPs (average of 1.23 SNPs per kb), and most of them (9,953, 97.7 %) were located on non-coding sequences and fewer (237, 2.3 %) on coding sequences (Table S2). Among the 95 protein-encoding genes within the channel catfish SDR, we did not find any obvious candidate MSD gene apart from *bcar1*, which has been recently described as the potential channel catfish MSD gene (BIB). This *bcar1* gene was localized on the edge of the Y chromosome non-recombinant region in a region with one male-biased SNP (see Figure S6). No male-biased SNPs were detected in the coding region of the *bcar1* gene but the first intron (Supplemental excel file 2). However, this male-biased SNP was not observed in the cultured population. It indicated that this SNP was likely group-specific in the wild population. In addition, 32 genes expressed higher in males than females were identified during the early stage of SD previously (Bao et al., 2019). But only one gene, *sphkap*, is linked tightly with some of our male-specific insertions.

4. Discussion

Channel catfish, belonging to the Ictaluridae family, is a native species from North America, and was introduced worldwide for recreational fishing and aquaculture. The understanding of its SD mechanism would not only benefit research on the evolution of SD in vertebrates but will have also an important impact on aquaculture practice. In teleost, GSD systems include both male heterogametic (XX/XY) and female heterogametic (ZZ/ZW) for example, goldfish (Wen et al., 2020), rainbow trout (Yano et al., 2012), and medaka (Matsuda et al., 2002) that have a male heterogametic SD system, or Chinese half-smooth tongue sole that has a female SD heterogametic system (Cui et al.,

Table 1
Summary of the male and female channel catfish pool-sequencing datasets.

Sample	Clean reads	Clean reads (Gb)	Mapped ratio (%)	Q20 (%)	Q30 (%)	GC (%)	Depth (x)
Male pool	266,697,484	40.3	98.5	96.7 %	91.9 %	40	34
Female pool	231,722,384	35.0	98.3	96.8 %	92.0 %	40	30

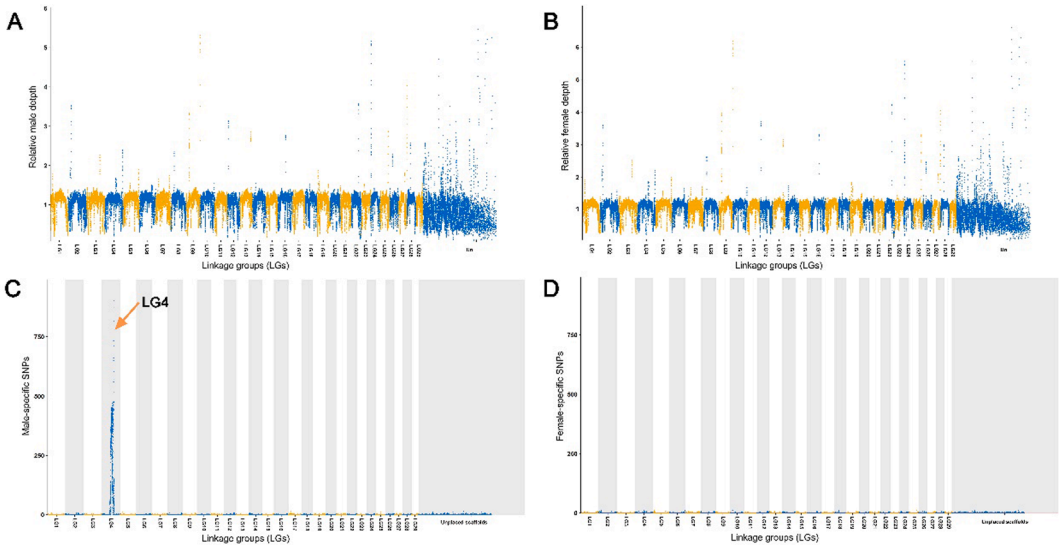

Fig. 1. Pool-sequencing results of male and female identify a male heterogametic system with a large sex determination region on LG04 using YY channel fish as reference. A 100 kb sliding window with an output point every 10 kb was used to calculate overall SNPs and depth. All the 29 linkage groups (LGs) were labelled with their LG number with all unplaced scaffolds combined together. (**A**) The distribution of relative male depth. (**B**) The distribution of relative female depth. (**C**) The distribution of male-SNPs showing a region with enriched male-specific SNPs on LG4. (**D**) The distribution of female-SNPs.

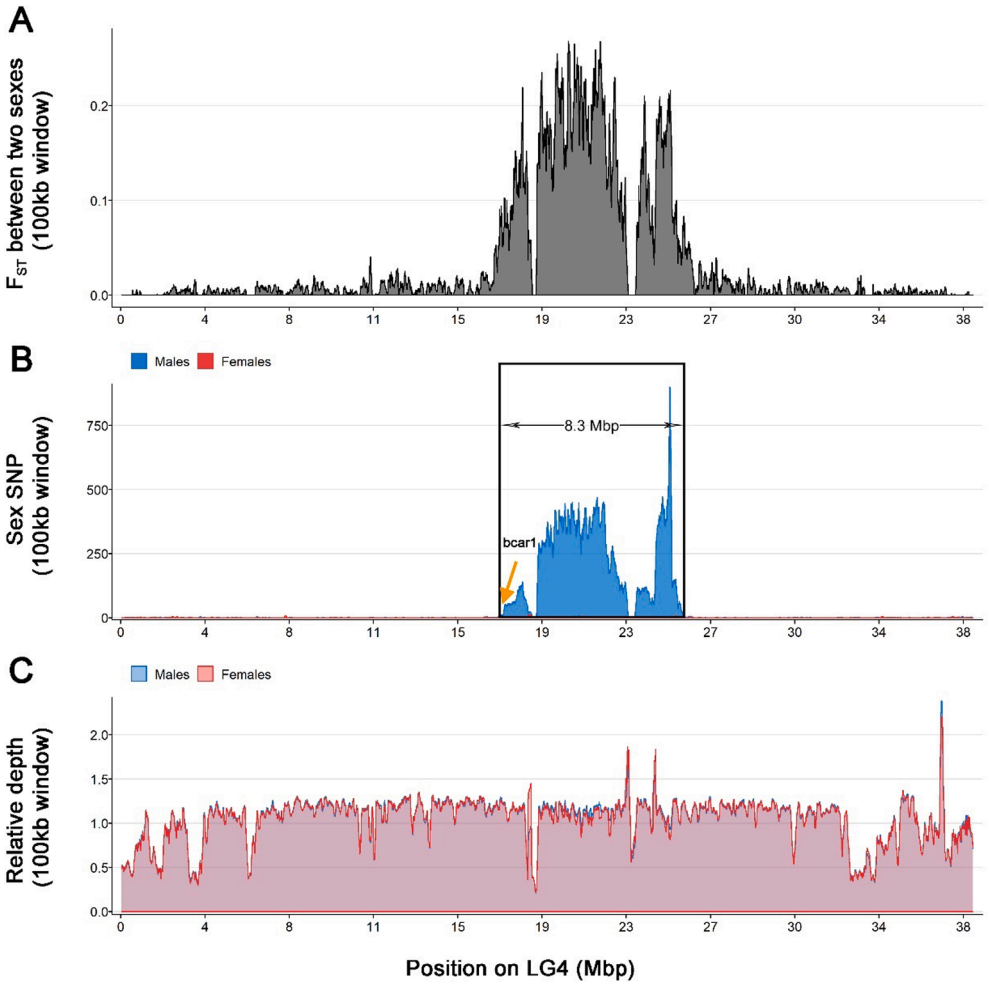

Fig. 2. Characterization of sex chromosome and sex-locus in channel catfish. Overall F_{ST} (**A**), sex-specific SNPs (**B**) and male / female relative read depth (**C**) were calculated using a 100 kb sliding window with an output point every 10 kb. A significant sex-biased signal with a high F_{ST} was observed on LG04 within a 8.3 Mb sex determining region (SR, highlighted with a black box on (**B**)) characterized by high density of male-specific SNPs (**B**). The *bcar1* gene on the edge of the SDR is shown by an orange arrow. Female- and male-specific SNPs are respectively indicated by red and blue color. (**C**) Relative read depth ratio between male (blue) and female (red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

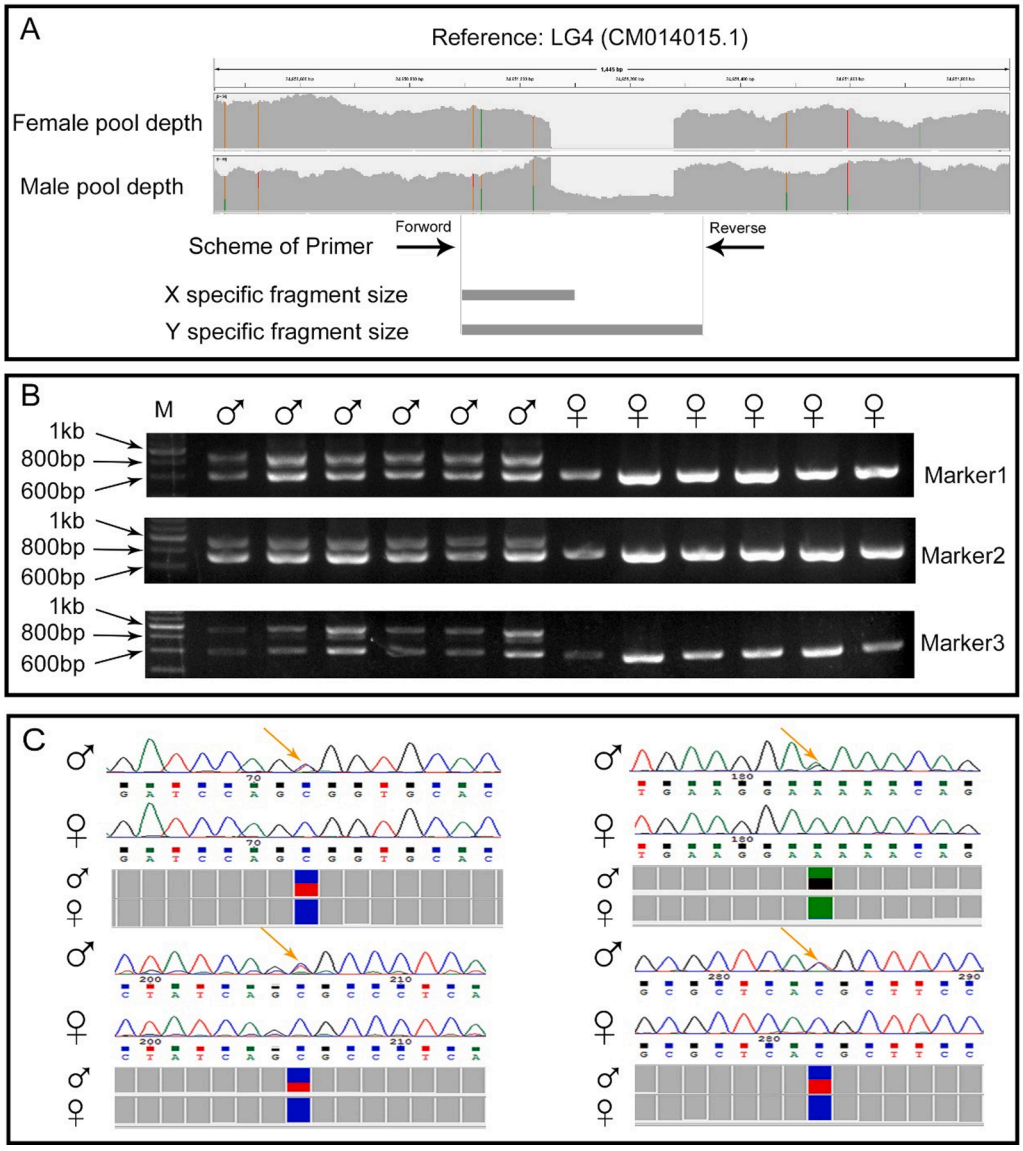

Fig. 3. Sex specific fragments and sex-specific polymorphic nucleotides. (A) Male specific fragments were observed within/around SDR with no/few reads mapped in female pool and half average depth in male pool. Sex-specific primers were designed by spanning the male-specific fragments, generating different size PCR products between male and female. (**B**) PCR validation of three sex-specific markers presenting in agarose gel with 6 males (♂, left side) and 6 females (♀, right side). The additional analysis on 12 males and 26 females was shown in Figure S5. (**C**) Random male-specific SNPs from SDR were manually checked using IGV, and verified by Sanger sequencing.

2017). Channel catfish is known as a species with a male SD heterogametic system (Patiño et al., 1996). In this study, using a novel pool-sequencing approach, we confirmed previous results (Bao et al., 2019) showing that this species is male heterogametic with a large non-recombining SDR on LG04. Pool-sequencing is increasingly applied for searching population genomic polymorphisms (Schlötterer et al., 2014), which is cost-efficient and quite practical in identifying genetic differences between populations with trait dimorphism. Reasonably, this sequencing strategy has been efficiently and successfully applied in characterizing SD in teleost fish with a strict GSD system (Feron et al., 2020; Wen et al., 2020; Pan et al., 2019; Gammerdinger et al., 2016). In channel catfish; environmental factors including temperature, like in goldfish (Goto-Kazeto et al., 2006) and Nile tilapia (Wessels and Hörstgen-Schwark, 2011), have been shown to interfere with the GSD system, thus preventing the use of a pooled strategy that can be biased by sex-reversed individuals (Patiño et al., 1996). This influence of temperature on channel catfish sex-differentiation has been shown to produce male-to-female sex reversals (Thorvaldsdottir et al., 2013) that would result in XY females in species with a male heterogametic SD system. These sexes reversed XY females, depending on their proportion in a pool of phenotypic females, could prevent the characterization of a sex-biased signal using a pooled strategy like the one we used. However, such sex-reversal is only induced at a high temperature (34°c) for channel catfish that is unlikely to be observed in the wild-type population that we sampled within its native range of distribution (Wisconsin, USA). The results of our whole genome Pool-Seq analysis and the individual genotyping of additional individuals confirmed the absence of such male-to-female sex reversions in our wild-type population. This suggests that channel catfish SD is mainly driven by a GSD system at least in its native habitats. It would be however interesting to explore further different aquaculture populations to investigate if captive breeding conditions could produce male-to-female sex reversals.

Growth in channel catfish has been described as sexually dimorphic with males growing faster than females (Simco et al., 1989). Therefore, the establishment of an all-male population of channel catfish would increase production in commercial aquaculture operations. All-male or all-female fish populations can be generated through gynogenesis or the production of special sex-reversed breeders using hormonal sex control (Hendry et al., 2003). The optimal strategies to produce a monosex population are variable depending on the desired sex phenotype and the GSD system of the species. Genetic tools such as sex markers could simplify and shorten these procedures by quickly identifying special phenotype/genotype associations (Yano et al., 2012; Cui et al., 2017; Dan et al., 2013) for instance potential XY females or even YY males that

would be sought after for the production of an all-male population in channel catfish aquaculture. The characterization of sex-specific variations for the development of a genetic sexing method like the one we provide here in channel catfish are then of great interest for their application for better sex control in aquaculture. Compared to one pair of sex-specific marker with small products size developed previously (Bao et al., 2019), our primers can generate bigger product size and the size difference between the two bands generated in males are significant enough, which is helpful to reduce false positive in genotyping. As our genotyping approach in channel catfish likely allows the identification of both X- and Y-alleles, different genotypes (sex XX, XY, and YY) can be potentially characterized. Therefore, it is possible to generate YY fish by combining temperature or steroid-induced sex reversals to first produce XY females that can be crossed with a normal XY male to produce YY males that are needed to generate genetically all-male populations. Both XY females and YY males can be likely detected with our sex genotyping tests, allowing a much quicker genotyping than the progeny testing protocols that were used without such genetic sexing tools.

Mammals and most birds have cytologically differentiated sex chromosomes. In mammals, the Y chromosome is highly degenerated and contains large repetitive sequences, small pseudo-autosomal regions and very few protein-encoding genes compared to the X chromosome. In teleost fishes, most species do not have morphologically differentiated sex chromosomes that can be distinguished on simple metaphase chromosome karyotypes (Bao et al., 2019). However, this does not mean that these morphologically undifferentiated sex chromosomes could not have a large differentiated SDR at the molecular level. For instance, relatively large SDR was reported in species with morphologically undifferentiated sex chromosomes, for instance in goldfish (SDR ~ 11.7 Mb) (Wen et al., 2020); blackchin tilapia, *Sarotherodon melanotheron* (SDR ~ 17.9 Mb) (Gammerdinger et al., 2016); and Nile tilapia *Oreochromis niloticus* (~10.7 Mb) (Gammerdinger et al., 2014). In contrast, small SDRs were identified through whole genome analysis in yellow perch, *Perca flavescens* (SDR ~ 0.1 Mb) (Feron et al., 2020), and common seadragon, *Phyllopteryx taeniolatus* (SDR ~ 47 kb) (Qu et al., 2021). In the Takifugu; *Takifugu rubripes* (Kamiya et al., 2012); the SDR is restricted to the minimum size with only a missense SNP on the Y chromosome associated with SD. In channel catfish, the Y chromosome (38.5 Mb) is slightly longer than the X chromosome (34.5 Mb), and we identified a relative large SDR (8.3 Mb) on LG4 as previously described using the whole genome sequencing of a YY male (Bao et al., 2019). This region is highly enriched with male-specific SNPs; male-specific insertions, and many chromosomal rearrangements between X and Y which can suppress recombination between X and Y chromosomes (Ponnikas et al., 2018).

A high diversity of MSD genes have been reported in teleosts (Pan et al., 2016), and most of these genes belong to a few gene families known as "usual suspects" MSD genes (Herpin and Schartl, 2015) like the Doublesex/Mab3 (DM) domain family (Matsuda et al., 2002; Cui et al., 2017; Nanda et al., 2002), transforming growth factor beta (TGF-β) family (Wen et al., 2022; Feron et al., 2020; Pan et al., 2019; Qu et al., 2021; Kamiya et al., 2012; Reichwald et al., 2015; Rondeau et al., 2013; Myosho et al., 2012; Hattori et al., 2012); steroid enzymes (Sandra and Norma, 2010); and SRY-related HMG-Box (Sox) family (Takehana et al., 2014). However, exceptions to this "usual suspect" rule have been found such as the *sdY* in rainbow trout (Yano et al., 2012), and *paics* in blue tilapia, *Oreochromis aureus* (Tao et al., 2021). In channel catfish, *bcar1*, a member of the Crk-associated substrate (CAS) family of scaffold proteins, was strongly suggested as an MSD gene due to its location within the SDR, the expression of a male-specific isoform differentiating male gonads, and the male-to-female sex reversal of *bcar1* knockout males (Bao et al., 2019). In our study, we found that one sex-biased SNP in the *bcar1* locus was localized in the first intron and that *bcar1* is surprisingly located at the edge of the SDR with relatively few male-specific SNPs in the wild-type. However, this sex-biased SNP was absent in the cultured population, implied it was group-specific. There were no sex-biased SNPs observed on the genomic sequence of *bcar1* corresponding to previous results (Bao et al., 2019). Most the reported SD genes originated from allelic diversification or gene duplication (Herpin and Schartl, 2015). However, bcar1 as an SD gene was not derived from both of them, but evolved as gonad specific transcript for initializing early gonad to differentiate to either testis or ovary (Bao et al., 2019). The gene, *sphkap*, expressed higher in males than females and linked tightly with some of our male-specific insertions in the channel catfish, which was also sexually dimorphic expressed during the earliest stages of gonad development in mouse (Bouma et al., 2010). It implies that it is possible to exert role on SD in channel catfish to some extent, but it needs further functional experiments to be validated.

5. Conclusions

Our results confirmed that channel catfish has a male heterogametic (XX/XY) system, and SD in that species is strongly regulated by genetic factors. Using a complimentary approach than those previously used (Wen et al., 2020; Pan et al., 2019), a large non-recombining region (~8.3 Mb) was characterized on LG04 of channel catfish, confirming the size of this SDR region and that LG04 is the sex chromosome of this species. The already described channel catfish *bcar1* MSD gene was also found in this large non-recombining LG04 region but surprisingly in a region with a relatively low density of male-specific SNPs, on the edge of this large SDR. There are no genomic differences observed at *bcar1* coding sequences. In addition, we used some small male- and Y-specific insertions in this SDR region to develop simple PCR-based sex genotyping tools that enable the identification of XX, XY, and YY genotypes, that can be now used for the production of all-male populations in channel catfish aquaculture.

Data availability

Male and female channel catfish pool-sequencing reads have been deposited in the Sequence Read Archive (SRA), under BioProject PRJNA821372.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gene.2022.146933.

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