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Characterization of Microsatellite Distribution in Siamese Fighting Fish Genome to Promote Conservation and Genetic Diversity

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Abstract: The Siamese fighting fish (*Betta splendens*) is a well-known ornamental fish and emerging model species for studying animal morphology, physiology, and behavior. A key concern of betta inbreeding is the decline in genetic diversity resulting from commercial breeding programs. Therefore, it is essential to develop markers for understanding the genetic bases of the domestication and phenotypic diversification of this species. We utilized the previously assembled genome of Siamese fighting fish to identify and characterize microsatellites and compare their genomic organization across different species. We annotated 812,134 microsatellite loci spanning 30.70 Mb, accounting for 6.57% of the Siamese fighting fish genome. We performed in silico polymorphism screening of microsatellites in the Siamese fighting fish and related species and present these sequences as candidate markers for cross-species amplification. In addition, we successfully validated two microsatellite loci using PCR-based assays in different species, which can promote further genetic characterization of diverse betta lineages. The set of polymorphic markers identified in this study may facilitate the assessment of genetic diversity and population structure and marker-assisted selection, among other applications.

Keywords: betta; microsatellite; genome; diversity; sex chromosome; transferability

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1. Introduction

Fighting fish or betta (*Betta* spp.) is a popular pet fish that is native to Southeast Asia and well known for its ornamental attributes [1]. Of the 91 identified species of fighting fish, the most well-known is the Siamese fighting fish (*Betta splendens*, Regan, 1910) [2]. The Siamese fighting fish is an emerging model species in biology and is economically important as an ornamental fish. This species is also subject to conservation concerns [1,3–5]. Features such as the species' unique body color pattern, scale iridescence,

body shape, and fin size have promoted a flourishing global market for Siamese fighting fish [1,3]. Changing the breeding patterns of the species following ornamental trends is preferable for a commercial strategy to generate higher profits for local sale and export. Those with novel body and fin color and shape patterns are mostly demanded for customers around the world. As a result, the species has been cross-bred to establish a niche market for ornamental fish, and wide varieties in morphology, pigmentation, body size, and fin shape have emerged during captive breeding that exhibit remarkable genetic differences from wild populations [6–9]. One limitation of captive breeding is that it limits the effective population of a species to the number of captive individuals that are available to interbreed. Limited effective population size in the captive breeding results in increased inbreeding that can deplete the viability of cultured fish [1,8,10,11]. The cultured fish that are greatly different from wild populations or those significantly inbred can be potentially threatening for autochthonic fish when escape from breeding farms. Moreover, widespread escapes of fish from breeding farms and artificial selection activities have resulted in outbreeding depression in various wild populations as well as significant inbreeding in commercial breeds of this species. Therefore, developing microsatellite markers to monitor the genetic condition of the wild Siamese fighting fish populations and to carry out marker-assisted selection (MAS) of broodstocks is necessary to effectively conserve the species' biodiversity.

DNA polymorphisms can be detected using various molecular markers, such as random amplified polymorphic DNA, restriction fragment length polymorphism, microsatellites, and mitochondrial DNA markers (*COI* and *Cytb*) [12–22]. Microsatellites are polymorphic loci derived from the repetition of short sequence motifs of 1–6 bp in length. They occur at thousands of locations within an organism's genome, including both coding and noncoding regions [23]. The advantages of microsatellite markers over other markers include high reproducibility, co-dominant transmission, high polymorphism, and multi-allelic nature [24–27]. In fighting fish, although DNA polymorphisms have been identified in different species using microsatellite markers to facilitate species-level genetic diversity analysis, a large portion of these sequences remains unexplored [8,14,28]. Next-generation sequencing (NGS) has provided excellent opportunities for biodiversity and breeding programs [29–32]. NGS is an effective method for detecting a large number of DNA markers such as single nucleotide polymorphisms (SNPs) in a short period based on diversity arrays technology (DArTseq™) and restriction-site associated DNA sequencing (RAD-seq) [7,8,33]. However, NGS-based genotyping is expensive and laborious, which limits its applicability in biodiversity conservation and local breeding programs. Farmers breeding small numbers of Siamese fighting fish may not have the budget for utilizing NGS to identify a large number of markers. In this context, microsatellite markers, as relatively inexpensive, can be used by small laboratories for genotyping Siamese fighting fish. The characterization of genome-wide microsatellites provides an economical approach for improving conservation and breeding. Microsatellites as markers are a tool for assessing genetic diversity, genetic map construction, comparative genomics, and MAS. Therefore, microsatellite markers contribute significant value in genotyping research and industry.

Microsatellite regions have a higher mutation rate than other areas of genome, they are characterized by a high level of polymorphism and have been applied in DNA fingerprinting, genetic diversity analysis, population structure analysis, and linkage mapping [8,14,34–36]. Genome-wide coverage and low requirements for expertise with instrumentation are attractive features of microsatellite genotyping. The traditional method for microsatellite isolation is expensive and time-consuming, involving the construction of microsatellite-enriched genome libraries, cloning, and sequencing using the Sanger method [14]. Another classical approach involves identifying microsatellite repeat motifs in DNA databases, such as expressed sequence tag sites [37,38]. The advent of NGS technologies and high-throughput whole-genome sequencing (WGS) has offered a faster and more cost-effective approach for genotyping by facilitating the detection of thousands of microsatellite loci in the genome of a target or non-model species [39]. Therefore, NGS is now frequently used in animal genetic diversity studies for identifying neutral markers such as microsatellites [40,41].

Several recent bioinformatics tools, such as Krait [42], MISA-web [43], and WebSat [44] have adapted and improved methods to identify microsatellite regions and developed markers using reference assemblies and WGS data. Reference genome assemblies and resequencing reads facilitate the identification and profiling of a large number of genome-wide polymorphic microsatellite markers. Chromosome-level genome assembly data of Siamese fighting fish (accession no: GCA_900634795.3) are available, which enables the identification and development of microsatellite markers [45–48]. In this study, we aimed to explore the genome of Siamese fighting fish to identify microsatellite-containing regions and to develop a set of polymorphic microsatellite markers via in silico genotyping. We further aimed to validate a subset of markers following PCR amplification and fragment sizing with small wild Siamese fighting fish populations. The validated markers were then evaluated for cross-species transferability to other fighting fish species using both in silico genome sequence comparison and PCR-based assays. These markers may facilitate the assessment of genetic diversity, population structure, MAS, and other applications.

2. Materials and Methods

2.1. Genome Sequences for Microsatellite Identification

Genome sequences of Siamese fighting fish (accession no: GCA_900634795.3, BioProject: PRJEB30365) were obtained from NCBI (http://www.ncbi.nlm.nih.gov/) in FASTA format (accessed on 3 June 2022). The genome was assembled along 21 chromosomes, and the sequence of each chromosome was analyzed individually [49].

2.2. Characterization of Microsatellites in Siamese Fighting Fish Genome

Microsatellites in the Siamese fighting fish genome were identified using Krait version 1.3.3 with default parameters of scanning for perfect, imperfect, and compound microsatellites [42] (Figure S1). The minimum repeat numbers for each perfect microsatellite were set to 12, 7, 5, 4, 4, and 4 for mono-, di-, tri-, tetra-, penta-, and hexa-nucleotides, respectively. The imperfect microsatellite selection criteria were as follows: (i) minimum sequence length and sequence repeat number were set to 8 bp and 3 times, (ii) maximum consecutive edits (including substitutions and indels) were specified as 3 bp, and (iii) the penalty cost was set to 1 for mismatch and 2 for indels (gaps), and the minimum required score to identify imperfect microsatellites was set to 10. For compound microsatellites, the maximum distance allowed between adjacent microsatellites was specified as 10 bp. To facilitate comparison among different repeat types, relative density (RD) and relative abundance (RA) as parameters were used for microsatellite analysis. Relative abundance (RA) indicates the number of microsatellites per Mb of the sequence analyzed, and relative density (RD) is the length (in bp) of microsatellites per Mb of the sequence analyzed. Krait v1.3.3 was applied to estimate the GC content and chromosome sequence size of Siamese fighting fish (accession no: GCA_900634795.3) [50]. Pearson's correlation coefficient was used to define correlations between variables, including the number of microsatellites, length of microsatellites, RA, RD, and chromosome sequence size. Correlation scatter plots and *p*-values were calculated using the 'ggscatter' function (from ggpubr package) in R version 4.2.0 [51]. The Circos program (http://circos.ca) (accessed on 20 June 2022) was used to draw Circos maps in which genome sequences were assembled into chromosomes. Microsatellites were mapped onto chromosomes using "circos.conf" files containing locus information [52], and microsatellite densities were identified as numbers in a sliding window of 3 Mb, with a step size of 100 kb. We also mapped microsatellites to different genomic regions (introns, intergenic regions, exons, and coding sequence (CDS)) using an annotation file (generic feature format: GFF) of Siamese fighting fish (accession no: GCA_900634795.3). To assign putative functions to the microsatellite-containing CDS region, the gene names of corresponding CDS-containing microsatellite loci were extracted. Gene Ontology (GO) analysis was then performed by functional classification using PANTHER (version 17.0) (http://www.pantherdb.org/) (accessed on 26 June 2022) setting zebrafish (*Danio rerio*, Hamilton, 1822) [53], Japanese rice fish (*Oryzias latipes*, Temminck and Schlegel, 1846) [54],

and spotted gar (*Lepisosteus oculatus*, Winchell, 1864) [55] as reference databases [56]. GO categorization was expressed as three independent hierarchies for biological processes, cellular components, and molecular functions.

2.3. Microsatellite Marker Development

A total of 10,000,000 reads (5,000,000 forward and 5,000,000 reverse reads) were extracted from the raw Illumina sequence of Siamese fighting fish (SRA: SRX14372870, BioProject: PRJNA778896) using seqtk toolkit and then used as the baseline data for marker development [50]. Microsatellite sequences were isolated from the extracted raw Illumina reads using QDD version 3.1 [57]. We focused on perfect microsatellites with high polymorphism, which exhibit significantly higher levels of genetic variation than other microsatellite types [58,59]. Mononucleotides were not considered because of the difficulty in distinguishing true microsatellites from sequencing or assembly errors [60]. Many microsatellites from raw Illumina sequences that contain reads do not have sufficient flanking sequences to allow PCR primer development [61–64]. We used the NCBI BLASTN program to blast microsatellites against the Siamese fighting fish genome with an additional 200 bp of flanking regions, in accordance with E-values < 0.005 and query coverage > 90% similarity. All markers were assigned to different Siamese fighting fish chromosomes (Table S1 and Figure S2). Oligonucleotide primers were designed using Primer3Plus [65] (accessed on 23 June 2022, https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). Default parameters to generate PCR products 100–400 bp in length had minimum, optimum, and maximum values of primer length (bp) 18–24 and Tm (°C) 50–60.

2.4. In Silico Polymorphism Screening of Siamese Fighting Fish Species

To evaluate the polymorphism level of developed microsatellite markers in silico, Siamese fighting fish genome sets were retrieved from the NCBI database (Table S2). The quality of Illumina reads was evaluated using FastQC [66], and the raw reads were trimmed to discard adapters using the Trimmomatic software V0.32 [67]. The cleaned reads were then aligned against sequences of the chromosome level genome assembly of Siamese fighting fish to investigate variations in the number of microsatellite repeat units within multiple individual genome accession numbers. The paired-end reads of each of the fighting fish accessions were mapped to the chromosome sequences of the Siamese fighting fish reference genome using BWA mem v.0.7.17 [68]. SAMtools v.1.9 [69] was used to convert SAM to BAM format, discard the unmapped reads, and sort and index the final alignment file. Mapping and in silico genotyping were performed at the Center for Agricultural Biotechnology (CAB), Kasetsart University. A bed file containing the chromosome name, start and end positions of microsatellite loci, motif length, number of repeat units in the reference sequence, and microsatellite locus name was created for each of the selected microsatellite loci received from QDD version 3.1 [57]. The aligned bam files of the 11 accessions of Siamese fighting fish were SRR18231392, SRR18231393, SRR18231394, SRR18231395, SRR18231396, SRR18231397, ERR4790883, ERR4790879, ERR4790878, ERR4790875, and ERR4790873, and the bed file contained the microsatellite regions in the reference genome. The reference chromosome sequences used to identify the microsatellites were implemented to alleles in the HipSTR program [70]. The HipSTR program was run using external stutter models and microsatellite calling in de novo allelic generation mode.

2.5. In Silico Cross-Species Transferability

The genomes of the following 16 fighting fish species were retrieved from the NCBI database: Mahachai betta (*B. mahachaiensis*, Kowasupat et al., 2012) [71], Mekong fighting fish (*B. smaragdina*, Ladiges, 1972) [72], peaceful betta (*B. imbellis*, Ladiges, 1975) [73], Toba betta (*B. rubra*, Perugia, 1893) [74], scorpion betta (*B. brownorum*, Witte and Schmidt, 1992) [75], spotfin betta (*B. macrostoma*, Regan, 1910) [2], Chukai betta (*B. tussyae*, Schaller, 1985) [76], betta pulchra (*B. pulchra*, Tan and Tan, 1996) [77], betta livida (*B. livida*, Ng and Kottelat, 1992) [78], betta burdigala (*B. burdigala*, Kottelat and Ng, 1994) [79], slim betta

(*B. bellica*, Sauvage, 1884) [80], betta compuncta (*B. compuncta*, Tan and Ng, 2006) [81], betta hipposideros (*B. hipposideros*, Ng and Kottelat, 1994) [82], betta ideii (*B. ideii*, Tan and Ng, 2006) [81], snakehead betta (*B. channoides*, Kottelat and Ng, 1994) [79], and betta mandor (*B. mandor*, Tan and Ng, 2006) [81] (Table S2).

All sequence management was performed using previously described bioinformatic methods to detect specific microsatellite loci. The aligned bam files of the 26 accessions (from 16 species) were performed using SAMtools mpileup and BCFtools [83] for variant calling, and the vcfutils.pl and vcf2fq commands (https://github.com/lh3/samtools/blob/master/bcftools/vcfutils.pl) were used to create consensus FASTQ files. The FASTQ files were then converted to FASTA files using the seqtk seq command. The FASTA files of the 26 accessions were subjected to in silico PCR analysis using Geneious software v2022.1.1 (Biomatters, Auckland, New Zealand; https://www.geneious.com) (accessed on 30 June 2022) to examine cross-species transferability in all designed primers (Table S1).

2.6. Specimen Collection and DNA Extraction

Twelve fighting fish species were collected from the wild in Thailand; details are presented in Table 1. All animal care and experimental procedures were approved by the Animal Experiment Committee of Kasetsart University, Thailand (approval no. ACKU63-SCI-007) and conducted in accordance with the regulations on animal experiments at Kasetsart University. Caudal fin clips were collected from each fish and preserved in 70% ethanol solution for DNA extraction. Total genomic DNA was isolated following the standard salting-out protocol as described previously [84] with slight modifications. DNA quality and concentration were determined by 1% agarose gel electrophoresis and spectrophotometry (NanoDrop One Microvolume UV-Vis Spectrophotometer, The Thermo Scientific™), respectively.

Table 1. Details of fighting fish used for microsatellite marker testing.

Species	Common Name	Breeding Type	Location	Latitude	Specimens
Betta splendens	Siamese fighting fish	Bubble nesting	Bangkok	13.7563° N, 100.5018° E	10
			Chumphon	10.4930° N, 99.1800° E	5
			Surat Thani	9.1342° N, 99.3334° E	6
Betta mahachaiensis	Mahachai betta	Bubble nesting	Samut Sakhon	13.5498° N, 100.2741° E	2
Betta siamorientalis	Eastern wild betta	Bubble nesting	Chachoengsao	13.6904° N, 101.0780° E	2
Betta imbellis	Peaceful betta	Bubble nesting	Krabi	8.0863° N, 98.9063° E	2
Betta smaragdina	Mekong fighting fish	Bubble nesting	Mukdahan	16.5436° N, 104.7024° E	2
Betta prima	Three-lined mouth-brooder	Mouth-brooders	Chanthaburi	12.6112° N, 102.1038° E	2
Betta simplex	Simple mouth-brooder	Mouth-brooders	Unknown	-	2
Betta pi	Pi betta	Mouth-brooders	Unknown	-	2
Betta pallida	Pallida betta	Mouth-brooders	Unknown	-	1
Betta apollon	Apollon betta	Mouth-brooders	Unknown	-	2
Betta ferox	Ferox betta	Mouth-brooders	Unknown	-	2
Betta pugnax	Penang betta	Mouth-brooders	Phatthalung	7.6167° N, 100.0740° E	2

2.7. Microsatellite Genotyping of Siamese Fighting Fish

Twelve developed microsatellite markers were used for test genotyping of Siamese fighting fish. The 5′-end of the forward primer of each set of primers was labeled with a fluorescent dye (6-FAM or HEX; Macrogen Inc., Seoul, Korea) (Table 2). PCR amplification was performed using 15 µL of 1× ThermoPol buffer containing 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 5.0 µM primers, 0.5 U *Taq* polymerase (Apsalagen Co., Ltd., Bangkok, Thailand), and 25 ng genomic DNA. The PCR protocol was as follows: initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 52–61 °C for 30 s, and extension at 72 °C for 30 s, with a final extension at 72 °C for 10 min. The PCR products were separated by electrophoresis on a 1% agarose gel. For each sample, PCR amplification was performed at least thrice to reduce the impact of erroneous alleles. Fluorescently labelled PCR amplification products were genotyped

using an ABI 3730XL automatic sequencer (Applied Biosystems, Foster City, CA, USA) at the DNA sequencing service of Macrogen, Inc. Allelic size was determined using the Peak Scanner version 1.0 software (Applied Biosystems, Foster City, CA, USA). The genotypic data generated in this study have been deposited in the Dryad Digital Repository (https://doi.org/10.5061/dryad.fbg79cnxq).

2.8. Microsatellite Marker Polymorphism Testing

The developed microsatellite markers were used to genotype 21 Siamese fighting fishes (*B. splendens*) (Table 2). The number of alleles (N_a), observed heterozygosity (H_o), and expected heterozygosity (H_e) were calculated using GenAlEx version 6.5 [85]. *PIC* was estimated using the Excel Microsatellite Toolkit [86]. For comparison with the previous eight microsatellite markers [14], we also genotyped the wild Siamese fighting fish population using PCR, as mentioned above.

2.9. Cross-Species Amplification Test of Microsatellite Markers

We genotyped 21 individuals belonging to 11 fighting fish species—Mahachai betta (*B. mahachaiensis*), Eastern wild betta (*B. siamorientalis*, Kowasupat, Panijpan, Ruenwongsa and Jeenthong, 2012) [87], peaceful betta (*B. imbellis*), Mekong fighting fish (*B. smaragdina*), three-lined mouth brooder (*B. prima*, Kottelat, 1994) [79], simple mouth brooder (*B. simplex*, Kottelat, 1994) [79], pi betta (*B. pi*, Tan, 1998) [88], pallida betta (*B. pallida*, Schindler and Schmidt, 2004 [89], apollon betta (*B. apollon*, Schindler and Schmidt, 2006) [90], ferox betta (*B. ferox*, Schindler and Schmidt, 2006) [90], and Penang betta (*B. pugnax*, Cantor, 1849) [91]—to investigate potential cross-species amplification of microsatellite markers (Table 1). PCR amplification for microsatellite genotyping was performed as mentioned above.

Table 2. Characteristics of microsatellite markers investigated in this study.

Locus	Primer Sequence (5′ to 3′)	Repeat	Ta (°C)	Size Range (bp)	Chromosome	Gene	Genomic Region	Source
BettaMS4	F: GTTTCATCAGGAGCAGCAGCATAA R: CTGTTTGATGGCCGACTTTT	(GA_n)	59	259–315	12	Noncoding gene	-	[13]
BettaMS5	F: GTTTCGTCACCTTCTGAGCAAACA R: AAATGCGCTGGGTAGACTTG	(GA_n)	59	198–218	3	*ush2a*	Between intron and exon	[13]
BettaMS8	F: CGTGAGCTGCAAAGAAAACA R: GCTGTTGCACATGAATCCAG	(GA_n)	57	223	14	*tcf7*	Intron	[13]
BettaMS15	F: ACTGTAACCGGGCTGTTCTG R: AACGCACCCAGAAACAAATC	(GA_n)	57	216–225	22	*dlgap2a*	Intron	[13]
BettaMS17	F: AAGCAGGTCTTTCACCTCCA R: TCACCCTGCGTCTAAGTCAA	(GA_n)	61	194–221	16	Noncoding gene	-	[13]
BettaMS23	F: GTTTGAGAGAAATGGGTTCTTCG R: TCACTACGCTGCCAAATCAG	(CT_n) (CA_n)	55	277–296	4	LOC114853122[+]	Intron	[13]
BettaMS25	F: GTTTGGGTAAAACCCAACTCTGG R: AACGTCACGTGGAACAGATG	(GT_n)	55	194–224	15	*ctu2*	Between intron and exon	[13]
BettaMS40	F: CAGTACATTTGACTGATCGCAGA R: CAGGATGCTTCCTTGGGTAA	(GA_n)	57	136–165	12	Noncoding gene	-	[13]
BettaMS10.1	F: TCTGAGGAAGGAGGCGATTA R: GCGTGCACTGAAGCATAAAG	(CA_n)	55	280–313	9	*slc20a2*	Between intron and exon	This study
BettaMS14.1	F: GGGCTGCACCTTAAACTCAT R: GTCCACTGGGCTGATGTTCT	$(TCCA)_n$	55	324–396	2	LOC114850832 [+]	Between intron and exon	This study
BettaMS2.2	F: ATTCCTTTCTGCCGCTAA R: AAAGAGGGCACTAAGCCA	$(TG)_n$	50	165–199	22	*meis2a*	Intron	This study
BettaMS14.2	F: CCCGGTTTCTTGTCATTC R: CGCTGATGGAAATTGAGT	$(TG)_n$	50	228	21	Noncoding gene	-	This study

[+] Uncharacterized gene.

3. Results

3.1. Microsatellite Distribution in Siamese Fighting Fish Genome

The total length of the Siamese fighting fish genome was 441,388,503 bp with a GC content of 45.3%. A total of 812,134 loci, consisting of 204,272 (72%), 585,320 (25%), and 22,542 (3%) perfect, imperfect, and compound microsatellite loci, respectively, were identified (Table 3, Figure 1). The microsatellites were frequent in the intronic region as 428,817 loci (52.80%). However, 41,588 microsatellite loci (5.12%) were located in functional gene regions, and almost all such microsatellite loci occurred in functional genes associated with cellular processes, binding, and cellular anatomical entities (Figure S3). The RA of perfect microsatellites was 462.82 loci/Mb, and the RD was 15,884.54 bp/Mb, while those of imperfect and compound microsatellites were 1,326.18 loci/Mb and 49,843.92 bp/Mb, respectively (Table 3). The most frequent motif of perfect and imperfect microsatellites was $(AC)_n$, whereas the most frequent motifs of compound microsatellites were $(CA)_n$–$(CA)_n$. Negative correlations were significantly observed between the frequency of microsatellite motifs and their length ($R = -0.83$, $p < 0.01$) (Figure 2). The average length of perfect microsatellite loci (total perfect microsatellite length/total perfect microsatellite counts) was approximately 34 bp, whereas that of imperfect microsatellite loci (total imperfect microsatellite length/total imperfect microsatellite counts) was approximately 38 bp, and that of compound microsatellite loci (total compound microsatellite length/total compound microsatellite counts) was approximately 75 bp (Table 3). Chromosome 4 was the largest (approximately 34.86 Mb) in the Siamese fighting fish genome, with a high proportion of microsatellite repeat motifs with perfect, imperfect, and compound microsatellites (16,333 loci, 569,518 bp; 45,851 loci, 1,747,106 bp; and 1,886 loci, 144,813 bp, respectively). The smallest chromosome was chromosome 12 (approximately 14.65 Mb), with perfect, imperfect, and compound microsatellites (6,918 loci, 240,601 bp; 19,617 loci, 745,206 bp; and 768 loci, 58,841 bp, respectively) (Tables S3–S5). The microsatellite count and length of all Siamese fighting fish chromosomes were strongly positively correlated with chromosome size (Figures S4 and S5).

Table 3. Summary of perfect, imperfect, and compound microsatellites detected in the whole genome of Siamese fighting fish (*Betta splendens*, Regan, 1910 [2] (accession no: GCA_900634795.3, 441 Mb).

	Description	Perfect Microsatellite	Imperfect Microsatellite	Compound Microsatellite
Total number of microsatellites	Unit	204,272	585,320	22,542
Total length of microsatellites	bp	7,010,790	21,999,074	1,692,553
Average of microsatellites	Total microsatellite length/total microsatellite count (bp)	34.33	37.58	75.08
Microsatellites per sequence	Total microsatellite counts/sequence counts	2918	8,362	322
Percentage of sequence covered by microsatellites	Total microsatellite length/total sequence length (%)	1.59	4.98	0.004
Relative abundance	Total microsatellites/total valid length (loci/Mb)	462.82	1326.18	51.07
Relative density	Total microsatellite length/total valid length (bp/Mb)	15,884.54	49,843.92	3,834.86

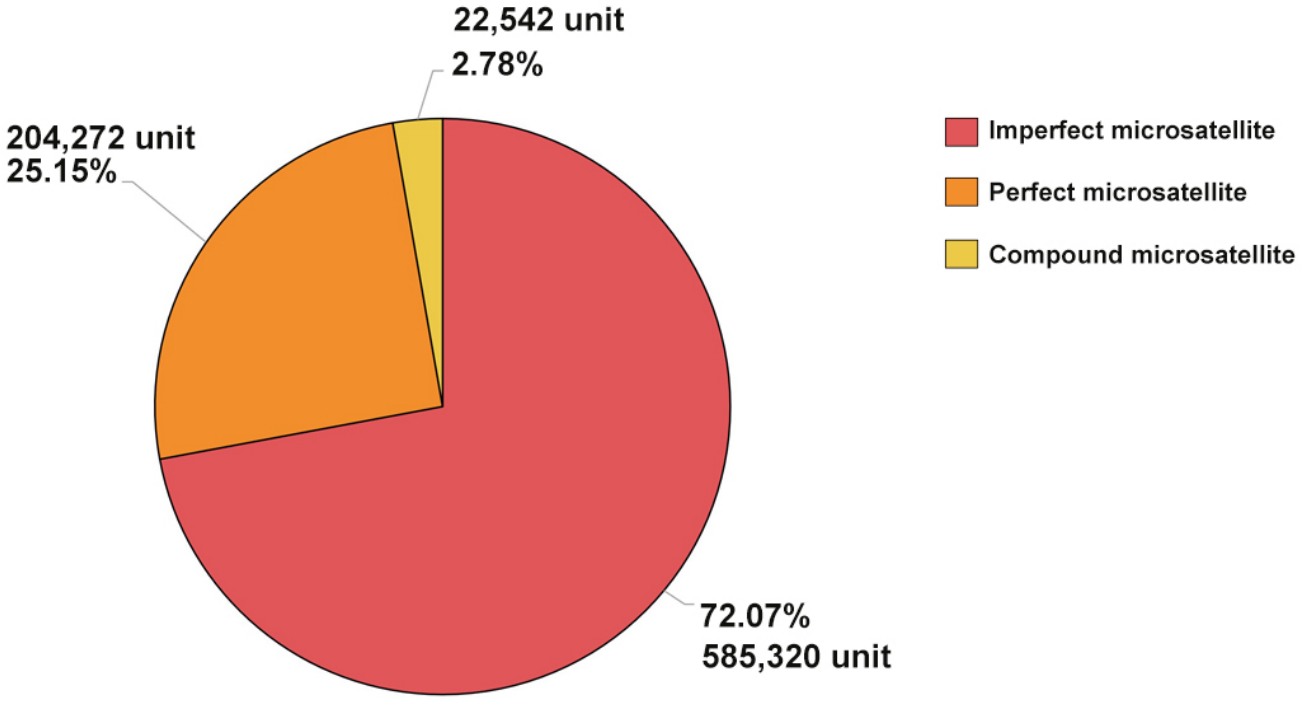

Figure 1. Pie diagram showing the portion of each microsatellite type in the Siamese fighting fish genome (*Betta splendens*, Regan, 1910) [2] (Tables S3–S5).

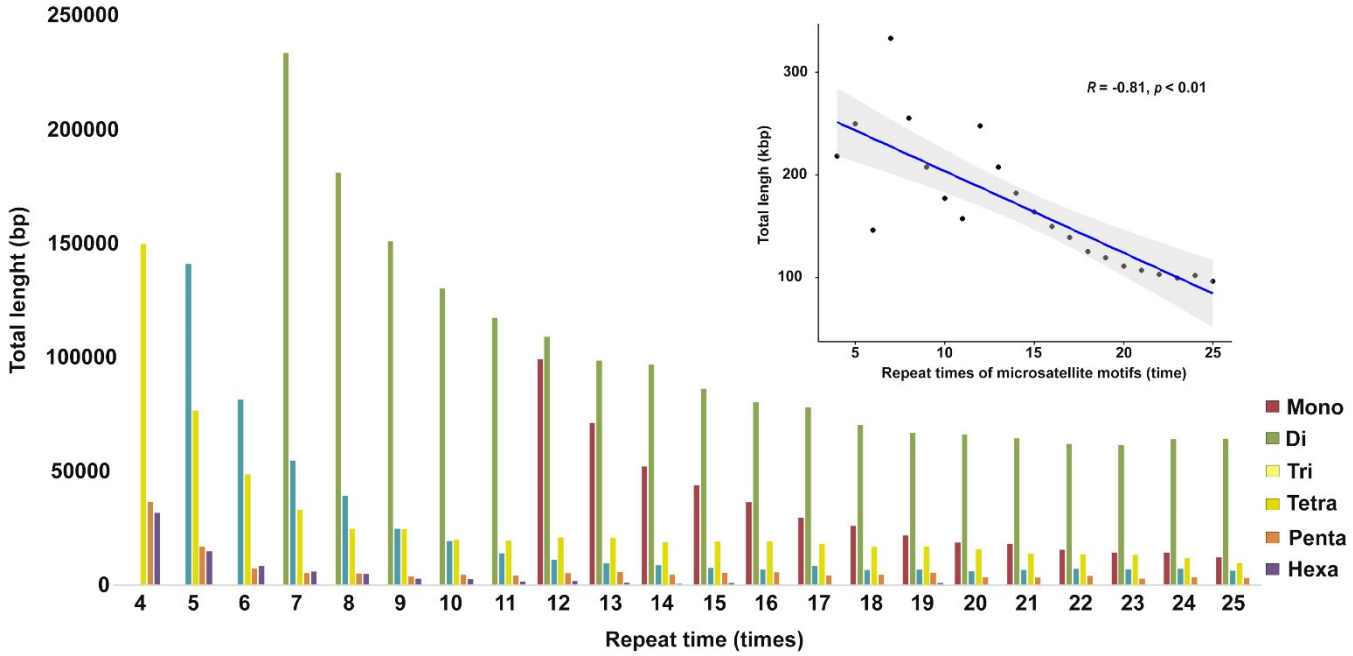

Figure 2. Scatter plots showing correlation (*R*) between the frequency of microsatellite motifs and their lengths.

The RD of perfect, imperfect, and compound microsatellites displayed on all chromosomes (Figure 3). The RA of perfect microsatellites ranged from 407.99 loci/Mb on chromosome 2 to 516.04 loci/Mb on chromosome 11, whereas the RD of perfect microsatellites ranged from 13,415.20 bp/Mb on chromosome 18 to 17,984.98 bp/Mb on chromosome 1 (Table S6). The lengths of all chromosomes were not correlated with the RA and RD of perfect microsatellites. Similar patterns were observed in the case of imperfect and compound microsatellites (Figures S6 and S7).

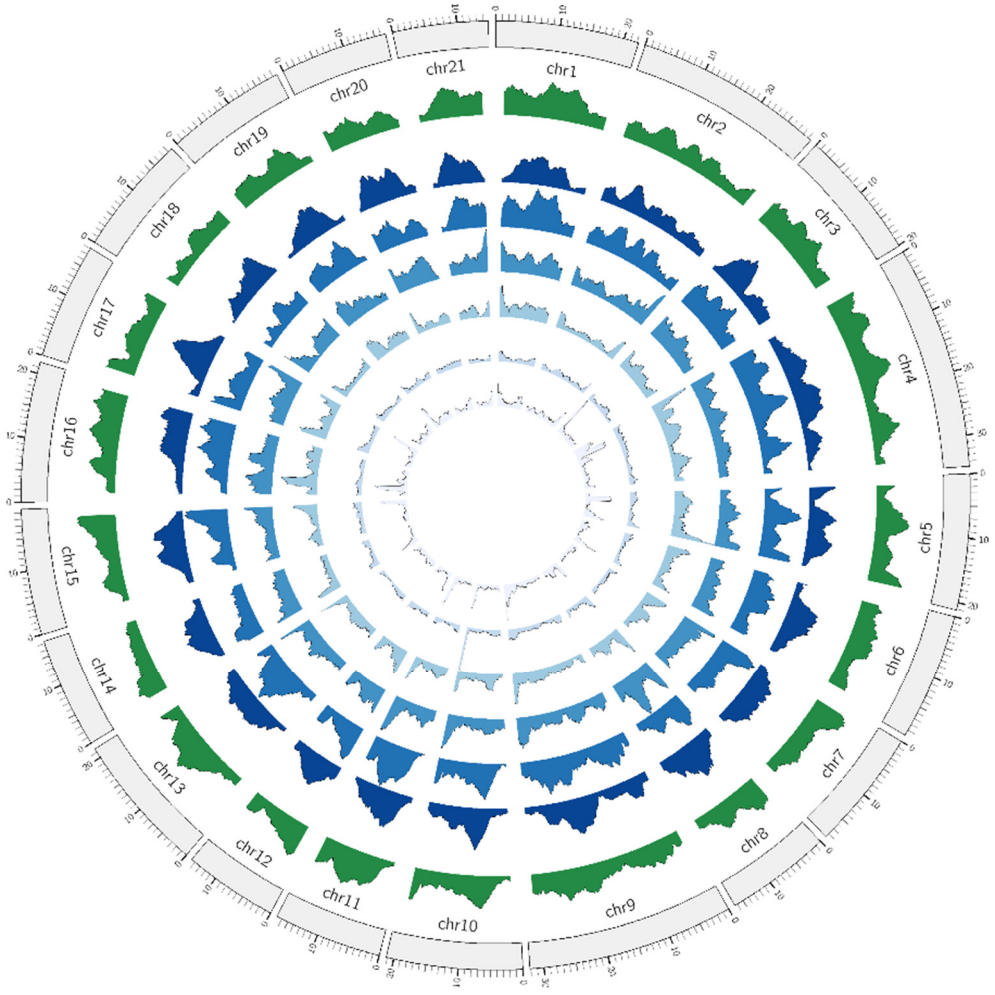

Figure 3. Association analysis of perfect microsatellites in the Siamese fighting fish genome. Circos was used to plot the assembled chromosomes and perfect microsatellite density. The outermost layer shows the chromosomes with numbers indicating the length in Mb. The dark green layer indicates the distribution of perfect microsatellites in 3 Mb windows with 100 kb steps. The blue gradient layer indicates the distribution of perfect microsatellites from mono (darkest blue) to hexa (lightest blue) in 3 Mb windows with 100 kb steps.

3.2. Polymorphic Microsatellite Identification through In Silico Genome Sequence Comparison and a PCR-Based Assay

Candidate microsatellites for marker development were selected from intronic and exonic genome regions located on different chromosomes. Forty primer pairs were randomly selected from all data and subsequently examined for polymorphism using two approaches: in silico genome sequence comparison and a PCR-based assay. For in silico genome sequence comparison, 11 Siamese fighting fish genomes were retrieved from NCBI and subjected to homology searching for 40 markers using the HipSTR program (Table S1). Of 40 microsatellite loci, 16 (BettaMS2.1, BettaMS4.1, BettaMS6.1, BettaMS7.1, BettaMS8.1, BettaMS9.1, BettaMS14.1, BettaMS1.2, BettaMS3.2, BettaMS4.2, BettaMS5.2, BettaMS7.2, BettaMS9.2, BettaMS12.2, BettaMS14.2, and BettaMS18.2) were identified as polymorphic. Furthermore, 21 wild Siamese fighting fish from three different localities in Thailand were genotyped for 40 microsatellite markers using PCR-based assays to estimate of their polymorphism level. From these, four markers were successfully amplified with polymorphic allelic profiles, and 46 alleles were observed among the four loci with a mean number of alleles per locus of 11.500 ± 4.330 (Table 4). The PIC values of all Siamese fighting fish ranged from 0.000 to 0.939, H_o values ranged from 0.000 to 0.952 (mean ± standard error

[SE]:0.417 ± 0.203), and H_e values ranged from 0.000 to 0.942 (mean ± SE:0.667 ± 0.223) (Table 4). Marker-level descriptive statistics are presented in detail in Table S7.

Table 4. Descriptive statistics for microsatellite markers based on 21 Siamese fighting fish (*Betta splendens*, Regan, 1910) [2].

Experiment		N_a	H_o	H_e	*PIC*
This study	Mean	11.500	0.417	0.667	0.659
	S.E.	4.330	0.203	0.223	0.221
Chailertrit et al., 2014	Mean	11.125	0.570	0.779	0.762
	S.E.	1.529	0.093	0.068	0.070
Both	Mean	11.250	0.519	0.742	0.728
	S.E.	1.643	0.089	0.082	0.082

Number of alleles (N_a); allelic richness (H_o); expected heterozygosity (H_e); polymorphic information content (*PIC*).

3.3. In Silico Cross-Species Transferability and Cross-Species Amplification

The potential for cross-species amplification of the developed microsatellite markers was determined in silico in 16 fighting fish species with 26 accessions using 40 primer pairs, and 24.42% were successfully amplified. Most were species belonging to the bubble-nesting group (Table S8). Cross-species amplification was performed to determine the transferability of microsatellite markers using PCR-based assays, and 82.73% microsatellite markers were successfully amplified in 11 fighting fish species (Table S9).

4. Discussion

In this study, we examined 1–6 bp long motif microsatellites in the whole genome of Siamese fighting fish and analyzed their distribution in different genomic regions. We identified 812,134 microsatellite loci making up 6.57% of the whole genome. The distribution frequency of microsatellites or the RA (approximately 600–700 loci/Mb) estimated in Siamese fighting fish genome was comparable to those in the genomes of other teleosts but lower than those in the genomes of green anole (1336 loci/Mb), chicken (1206 loci/Mb), mouse (1862 loci/Mb), and humans (1439 loci/Mb) [92,93]. A negative correlation between microsatellite frequency and microsatellite length was also found as in other vertebrates [45,48]. Although it remains unclear whether variability in frequency and sequence length of microsatellites can reshape genomic dynamics, it is hypothesized that microsatellites with longer repeat lengths are likely highly variable and derived from mutations in which longer repeats have higher mutation rates with replication slippage, which leads to reduced stability [94,95]. Consistent with previous studies on Nile tilapia (*Oreochromis niloticus*, Linnaeus, 1758) [96], Japanese puffer (*Takifugu rubripes*, Temminck and Schlegel, 1850) [97], and zebrafish (*D. rerio*), dinucleotide repeats were the most abundant microsatellites, followed by mono-, tri-, tetra-, penta-, and hexa-repeats [93]. The most abundant mononucleotide, dinucleotide, and trinucleotide motifs were A, AC, and AAT, which are similar to those in Japanese rice fish, zebrafish, and Nile tilapia, but different from that seen in Mexican tetra (*Astyanax mexicanus*, De Filippi, 1853) [98] and coho salmon (*Oncorhynchus kisutch*, Walbaum, 1792) [99]. This dynamic distribution of different repeat motif in a species- and genomic-specific manner suggests that the differential abundance of mononucleotide, dinucleotide, and trinucleotide motifs is likely influenced by selective forces and mismatch repair systems during genome evolution in teleosts [100]. Interestingly, the microsatellite mononucleotide and dinucleotide repeats are highly dynamic with higher levels of frequency, sequence length variations, and mutation rate as compared to trinucleotide, tetranucleotide, and pentanucleotide repeats [93]. However, differences between species may also arise due to variations in search criteria, database size, and bioinformatics software tools used in different studies for microsatellite detection [101,102]. Although GO annotation of microsatellite-containing genes revealed many functional genes involved in various biological activities of Siamese fighting fish. However, we did not find

any specific functions with significant enrichment score of gene ontologies. This suggests that microsatellite repeats might be associated with diverse functions of biological process with no preferential tendency to accumulate nonspecific gene sets. This result agrees with noncoding regions, which generally contain more abundant microsatellites than coding regions [93].

4.1. Coincidence of Microsatellite Density and in Sex-Determining Regions of Siamese Fighting Fish

We found that in the Siamese fighting fish genome, chromosome size was significantly positively correlated with the number of microsatellites but negatively correlated with their RA and RD. This finding reflects those reported for spotted sea bass (*Lateolabrax maculatus*, Cuvier, 1828) [103], domesticated cattle (*Bos taurus*, Linnaeus, 1758) [96], water buffalo (*Bubalus bubalis*, Linnaeus, 1758) [96], wild yak (*Bos mutus*, Przewalski, 1883) [104], sheep (*Ovis aries*, Linnaeus, 1758) [96], goats (*Capra hircus*, Linnaeus, 1758) [96], and Tibetan antelope (*Pantholops hodgsonii*, Abel, 1826) [47,105,106]. Different repeat types were observed between chromosomes within the genome; however, the density of repeats varied among different chromosomes. Microsatellite density is generally higher in telomeres than in other regions, as observed in the human and mouse genomes [107,108]. Furthermore, microsatellite density is higher in autosomes than in the X chromosome in mammals [109]. This is caused by differential degrees of euchromatin and heterochromatin on different chromosomes. Chromosome 9 (a sex chromosome) is the second largest chromosome in Siamese fighting fish, and microsatellite density in Siamese fighting fish chromosome is comparable to that of autosomes [5]. Similar cases have been observed in the Japanese rice fish (*Oryzias latipes*) and zebrafish (*Danio rerio*) [110,111].

Novel satellite DNA may emerge to support the process by which sex chromosome differentiation occurs [8]. For example, the mammalian sex chromosomes X and Y show high differentiation, and large microsatellite amplification has been observed in the Y chromosome as the counterpart of X to accelerate differentiation [100,112]. Autosomes do not undergo chromosome differentiation. Large microsatellite repeat motif amplifications observed in Y or W sex chromosomes are frequently observed in many vertebrates showing high sex chromosome differentiation [113–116]. However, most teleost sex chromosomes, including those of Siamese fighting fish, are likely to be in the early stage of sex chromosome differentiation, and a few microsatellite accumulations are expected to appear.

A comparison of the draft genome assembly of Siamese fighting fish with the genome sequences of Japanese rice fish (*O. latipes*, OLA) and zebrafish (*D. rerio*, DRE) showed conserved chromosomal syntenies among the three species [8,48,110,111]. This genomic information provided a new perspective on the comparative genomics of teleosts, which in turn facilitated extensive comparison of genomic structures at the molecular level. The XX/XY sex chromosome type is represented in Siamese fighting fish as BSP chromosome 9 (BSP9) and Japanese rice fish (*O. latipes*) (OLA9), whereas the ZZ/ZW type is represented in zebrafish (*D. rerio*) (DRE4). Recent studies have shown that vertebrates have complex genetic networks triggering sexual differentiation and are composed of substantially different factors [113]. In particular, the significant diversity of master sex-determining genes that influences genetic hierarchies has become apparent. A well-known master sex-determining gene in fish, the *dmrt1* gene, has been previously mapped on both BSP9 and OLA9 [8,114]. Interestingly, our analysis revealed a high density of microsatellite distribution near the *dmrt1* locus, suggesting the possible role of microsatellite repeats in sex chromosome evolution (Figure 4). However, the colinear block of homology is probably different as a consequence of intrachromosomal rearrangements, such as inversion or segmental duplication during chromosome evolution (Figure 4) [115]. BSP9 contains a putative sex-determining region (pMDR) in the long arm of the chromosome (BSPq) [8], and a high microsatellite distribution was identified around this region and its neighboring region. This suggests that the segment of differentiation expands in the sex chromosomes of Siamese fighting fish lineages. Although DRE4 does not contain *dmrt1*, a high density

of microsatellites has been observed in DRE4q, which contains functional genes of sex development and large sites of sex-determining regions [116].

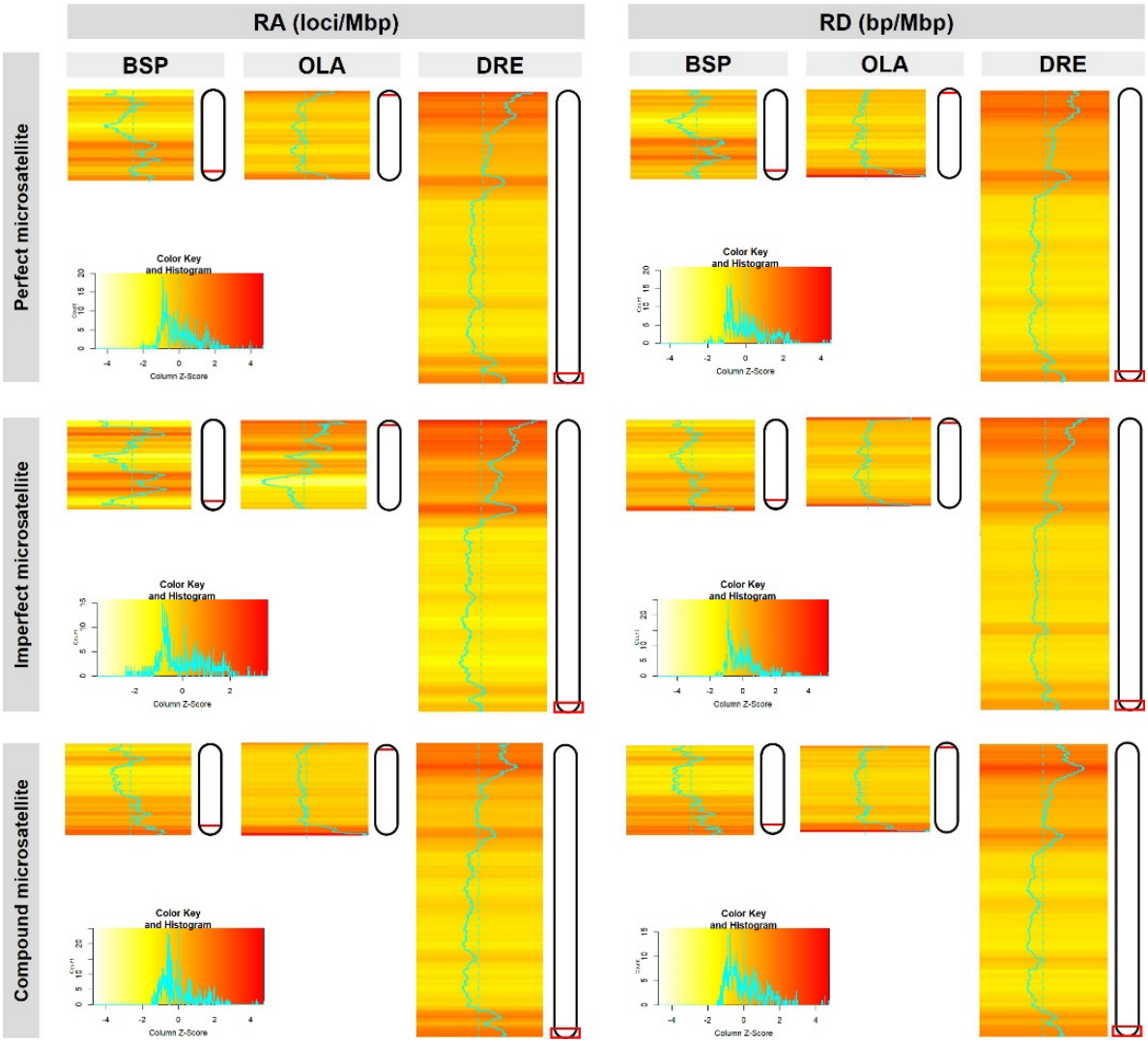

Figure 4. Microsatellite abundance and density in the sex chromosome of Siamese fighting fish (BSP) (*Betta splendens*, Regan, 1910, Chromosome 9) [2], Japanese rice fish (OLA) (*Oryzias latipes*, Temminck and Schlegel, 1846. Chromosome 9) [54], and zebrafish (DRE) (*Danio rerio*, Hamilton, 1822, Chromosome 4) [53].

4.2. Microsatellite Marker Development for Genetic Diversity Studies, Conservation, and Future Breeding Programs

The increasing intensity of anthropogenic activities, such as urbanization and industrial development, have compromised the habitats of Siamese fighting fish. Most wild fighting fish are currently listed as threatened according to the IUCN Red List [1,4,117,118]. The significant biodiversity of Siamese fighting fish is rapidly declining owing to the influx of invasive species or hybrids into the wild, leading to genetic admixture [1,10,11]. The development of reliable and effective approaches for monitoring the genetic diversity of fighting fish is urgently required for conservation management. Microsatellites are among the most useful markers for measuring the genetic diversity among species and have increased the potential of conservation genetics. However, a few polymorphic microsatellite markers are based on compound or imperfect motifs, which are difficult to interpret in routine genotyping assays because of allele binning difficulties [119,120]. Perfect microsatellite markers are more suitable, although they represent only a small fraction (approximately

2%) of the Siamese fighting fish genome. Currently, the use of microsatellite markers for Siamese fighting fish management is limited [14,28]. The identification and characterization of molecular markers is the first step in implementing microsatellite marker use for species management. Recent developments in NGS techniques have facilitated the de novo development of microsatellite loci; however, doing so is expensive, and subsequent polymorphism screening is time-consuming [121,122]. Obtaining good polymorphic loci requires further validation by PCR. In this study, 40 anonymous microsatellite markers were developed, of which 11 polymorphism markers were determined by in silico genome sequence comparison and four by PCR-based assay. Only two polymorphic microsatellite markers were successfully validated using these two approaches. The discrepancy in microsatellite polymorphisms between the two approaches may have resulted from the different Siamese fighting fish samples examined. The in silico genome sequence comparison was performed using ornamental commercial Siamese fighting fish available in the NCBI database (Table S2), whereas the PCR-based assay was performed with wild Siamese fighting fish from three localities. Xia et al. (2018) asserted that microsatellite repeat length was negatively correlated with the substitution rate in nearby flanking sequences, wherein a low substitution rate in nearby flanking sequences increased the rate of successful amplification [123]. Therefore, testing large and varied Siamese fighting fish populations is necessary to confirm the usefulness of microsatellite markers. The low polymorphism determined in our study may have resulted from reduced genetic differentiation of the Siamese fighting fish samples used in the screening or because the markers tested were located in more conserved regions of the genome.

More than 15–20 molecular markers are necessary to assess the genetic diversity in fish [124–127]. Polymorphic markers are often selected for subsequent studies; however, this might introduce ascertainment bias and generally overestimate genetic diversity [128,129]. Although we were able to screen hundreds of microsatellite loci using in silico method, more microsatellite markers must be identified from our sequence library to experimentally test polymorphisms in large and varied populations. Flanking sequences for all loci have been provided, and more primer sequences can be designed (Dryad Digital Repository. Dataset, https://doi.org/10.5061/dryad.fbg79cnxq). The flanking sequences can subsequently be used to redesign primers with different product sizes to fit multiplex runs. However, all microsatellite markers should be located on different linkages or chromosomes to avoid linkage disequilibrium. At minimum, four new polymorphic markers were developed from the Siamese fighting fish reference genome to add to the eight markers from previous studies, with average *PIC* values of more than 0.5 [14]. All markers were located on different chromosomes, namely, chromosomes 2, 3, 4, 9, 12, 14, 15, 16, 19, and 20. We also compared Siamese fighting fish microsatellite markers with those of other fighting fish species. Using in silico genome sequence comparisons, the percentage of cross-species transferability to other species of the bubble nesting group was high (33.59%), and it decreased in species belonging to the mouth brooders group (9.75%), consistent with the phylogenetic relationships of fighting fish [12,16–20]. This also suggests the possibility of chromosome synteny or linkage homology analysis. We identified more highly conserved syntenic blocks among the bubble nesting group than the mouth brooders group, further confirming the close evolutionary relationships and interspecific hybridization events. These blocks are necessary to identify large-scale intrachromosomal rearrangements in the same chromosome. Whether partial chromosome inversion affects genetic mapping and the study of some traits is worth exploring in the future. Similar results were observed when we performed cross-amplification PCR-based assays with other fighting fish. PCR products were obtained for at least 82.73% of the examined markers in all other fighting fish. This suggests that a substantial proportion of Siamese fighting fish microsatellite markers can be used in genetic studies on a wide range of fighting fish lineages. However, inherent problems such as allele size homoplasy, polymorphism bias, null allele presence, broken repeat motifs, or amplification of non-orthologous loci can arise, especially in studying

phylogenetically distantly related species [130–132]. De novo development of species–specific microsatellite markers is strongly recommended, especially for mouth-brooding fish.

5. Conclusions

This paper reports a genetic approach for monitoring the diversity and conservation of betta fish using molecular markers. We applied bioinformatic methods to detect novel microsatellite markers and investigate their genomic organization. To test the species-specific transferability of microsatellite markers, we used the PCR-based cross-species amplification and successfully validated two loci. The genomic-wide microsatellite characterization distribution provides evolutionary insights with species- and chromosome-specific dynamic distributions. Additionally, the prospective microsatellites highlighted in this study could be used in the genetic characterization of diverse betta lineages. Our approach of identifying polymorphic microsatellite markers will expedite the development of useful markers with known physical locations on chromosomes and avoid laborious preliminary molecular screening for polymorphisms.

Supplementary Materials: The following supporting information can be downloaded from: https://www.mdpi.com/article/10.3390/fishes7050251/s1. Figure S1: Types of microsatellites or simple sequence repeats: perfect microsatellite (a), imperfect microsatellite (b), and compound microsatellite (c). Figure S2: Pie chart showing the percentage of primer regions designed in this study (a) and bar chart of the primer loci number on Siamese fighter fish chromosomes (b). Figure S3: Gene ontology (GO) functional classification of genes containing perfect (a), imperfect (b), and compound (c) microsatellites. Figure S4: Scatter plots showing the correlation (*R*) between chromosome size and numbers of perfect (a), imperfect (b), and compound (c) microsatellites. Figure S5: Scatter plots showing correlation (*R*) between chromosome size and lengths of perfect (a), imperfect (b), and compound (c) microsatellites. Figure S6: Scatter plots showing correlation (*R*) between the total number of microsatellites and the relative abundances of perfect (a), imperfect (b), and compound (c) microsatellites. Figure S7: Scatter plots showing the correlation (*R*) between chromosome size and relative densities of perfect (a), imperfect (b), and compound (c) microsatellites. Table S1: Details of primers used in this study [133–144]. Table S2: Information on other fighting fish genomes. Table S3: Summary of perfect microsatellites in each Siamese fighting fish chromosome. Table S4: Summary of imperfect microsatellites in each Siamese fighting fish chromosome. Table S5: Summary of compound microsatellites in each Siamese fighting fish chromosome. Table S6: Details of perfect microsatellites in each Siamese fighting fish chromosome (*Betta splendens*, Regan, 1910) (accession no: GCA_900634795.3, 441 Mb). Table S7: Descriptive statistics at microsatellite markers level based on 21 Siamese fighting fish (*Betta splendens*, Regan, 1910). Table S8: In silico cross-species transferability of microsatellites markers in some fighting fish species. Table S9: Amplification of 11 species of the fighting fish using the microsatellite markers developed in this study.

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