

Article

Development of Novel SNP Assays for Genetic Analysis of Rare Minnow (*Gobiocypris rarus*) in a Successive Generation Closed Colony

Lei Cai ^{1,2,3}, Miaomiao Hou ^{1,2}, Chunsen Xu ^{1,2}, Zhijun Xia ^{1,2} and Jianwei Wang ^{1,4,*}

¹ The Key Laboratory of Aquatic Biodiversity and Conservation of Chinese Academy of Sciences, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430070, China; cailei17@163.com (L.C.); hougiao006@163.com (M.H.); 18227588944@163.com (C.X.); Xiazj1995@163.com (Z.X.)

² University of Chinese Academy of Sciences, Beijing 100049, China

³ Guangdong Provincial Key Laboratory of Laboratory Animals, Guangdong Laboratory Animals Monitoring Institute, Guangzhou 510663, China

⁴ National Aquatic Biological Resource Center, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430070, China

* Correspondence: wangjw@ihb.ac.cn

Received: 10 November 2020; Accepted: 11 December 2020; Published: 18 December 2020



Abstract: The complex genetic architecture of closed colonies during successive passages poses a significant challenge in the understanding of the genetic background. Research on the dynamic changes in genetic structure for the establishment of a new closed colony is limited. In this study, we developed 51 single nucleotide polymorphism (SNP) markers for the rare minnow (*Gobiocypris rarus*) and conducted genetic diversity and structure analyses in five successive generations of a closed colony using 20 SNPs. The range of mean H_o and H_e in five generations was 0.4547–0.4983 and 0.4445–0.4644, respectively. No significant differences were observed in the N_e , H_o , and H_e ($p > 0.05$) between the five closed colony generations, indicating well-maintained heterozygosity. The F -statistics analysis revealed a relatively stable genetic structure of the closed colony. Furthermore, the genetic distance between the newer and older generations increased with the breeding generations in closed colonies. Our results confirmed previous findings in the same samples using microsatellite markers. The results will be beneficial for establishing genetic variability monitoring criteria and restoration of the wild population of the rare minnow and other laboratory fish.

Keywords: rare minnow; closed colony; laboratory fish; genetic structure

1. Introduction

Research using laboratory animals requires explicit knowledge of their genetic background [1]. At present, technologies that monitor genetic variability in major closed colony organisms are mainly based on morphology, biochemical markers, or microsatellite markers (SSRs) [2–4]. Moreover, the genetic information provided by morphological and biochemical markers is limited; hence, they are not used to identify single base mutations. Owing to the difficulty in comprehensively evaluating the genetic background of laboratory animal, molecular marker technology has been introduced into the study of genetics, such as SSRs, which can provide sufficient genetic information, and have become available for detection of genetic diversity of laboratory animals [5]. However, as a third-generation DNA molecular marker technology and an excellent tool for population based genetic research [6,7], the research of using single nucleotide polymorphisms (SNPs) in genetic variability evaluation of closed colony is still inadequate. Advantages of SNPs include genetic stability, high polymorphism,

co-dominance, and suitability for high-throughput genotyping. SNPs are widely used for the evaluation of genetic diversity [8], research on population genetic variations [9], and analysis of disease susceptibility [10].

In genetic research on traditional laboratory animals (terrestrial animals), SNP markers are widely used. For instance, in the field of inbred strain identification and background characterization, Jackson Laboratory (USA) supplied a 200 SNP panel for 129 mouse inbred strain and sub-strain characterization [11], Charles River Laboratories (USA) provided a 128 SNP panel for mouse inbred strain background analysis and a 384 SNP panel for 128 inbred strain and sub-strain characterization [12], and Taconic Biosciences (USA) identified 2050 SNP markers for 21 inbred strain background characterization [12]. In closed colony genetic research, high-density SNP arrays were used in outbred mouse diversity analysis [13], whereas genome-wide SNPs were used in the analysis of inbreeding [14], linkage disequilibrium [15], and genetic variation [16] of outbred mice. As most commonly used traditional laboratory animals were established decades ago, it is difficult to obtain original samples, especially from the first several generations. Hence, few studies about the genetic structure analysis of successive generations in newly developed closed colonies were reported. Compared to terrestrial laboratory animals, most fish closed colonies were established more recently and may have preserved relatively complete original samples. Laboratory fish may be an excellent tool for analyzing the dynamic changes in genetic structure during the course of establishing a new closed colony.

Laboratory fish are important laboratory animals widely used in the fields of developmental biology, genetics, biological medicine, and environmental toxicology [17] owing to the following advantages: moderate body size, short sexual maturity period, easy genetic manipulation, strong reproductive capacity, short spawning interval, annual reproduction, and easy indoor rearing. Currently, the global use of laboratory fish is third only to rat (*Rattus norvegicus*) and mouse (*Mus musculus*) [18], and compared to terrestrial animals, laboratory fish are more conducive to the 3 R Principle (Reduction, Replacement, and Refinement) [19]. The international research community has established several closed colonies of fish like zebrafish (*Danio rerio*) [20], rare minnow (*Gobiocypris rarus*) [21], Japanese medaka (*Oryzias latipes*) [22], *Xiphophorus maculatus* [23], *Xiphophorus helleri* [23], *Poecilia reticulata* [24], and *Mugilogobius chulae* [25]. The lack of genetic research on laboratory fish is a matter of concern for their future applications. Hence, it is imperative to reinforce genetic research of laboratory fish using new methods such as SNP molecular markers.

Rare minnow (*Gobiocypris rarus*) is an excellent fish model for analyzing changes in genetic structure throughout the establishment of a new closed colony [26,27]. A rare minnow closed colony (IHB) was established in 2006 from 50 pairs of wild-caught fish originating from the Sichuan Province of China [28] and has reached the 11th generation. The breeding of IHB was performed by strictly following the modern classical closed colony breeding theory to avoid inbreeding [29], and at present, the pedigree and original samples of IHB are complete. Because of its sound ecological and biological characteristics of IHB like high fecundity and sensitivity to toxins, IHB is widely used in ecotoxicology research, particularly in risk assessment of chemicals, and is the only indigenous Chinese fish recommended by “The Guidelines for the Testing of Chemicals” (Ministry of Ecology and Environment of China) [21]. As a newly emerging laboratory fish, there is a lack of genetic background research on IHB. The genomic SNPs in IHB are unknown, and genetic analysis using SNPs of successive generations of a closed colony in laboratory fish has not been reported.

In this study, we used bioinformatic methods to identify SNP markers and then validated the SNPs using SNaPshot methods [30]. Furthermore, we selected highly polymorphic SNPs to characterize successive generations of IHB and analyzed the changes in genetic structure and diversity between generations. We also compared the differences in the results of SNP and SSR marker analyses in the same samples. Our results may provide useful information to establish genetic variability control standards for rare minnow and may serve as a beneficial reference for other laboratory fish.

2. Materials and Methods

2.1. Fish Sampling

Adult male or female IHB (body mass: 0.99 ± 0.14 g) were collected from the fish facility at the Institute of Hydrobiology, Chinese Academy of Sciences, China. The population has been maintained as a closed colony for 11 generations. Thirty samples from F1, F3, F4, and F5 generations, and 32 samples from the F10 generation were collected. Six wild samples of the rare minnow were collected from Hanyuan, Sichuan Province, China. The study was approved by the Institutional Animal Care and Use Committee (IACUC) of the Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan.

2.2. DNA Extraction

Genomic DNA was extracted from fin samples using a tissue genomic DNA isolation kit (Tiangen, Beijing, China) following the manufacturer's instructions. The extracted genomic DNA was quantified using Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA).

2.3. SNP Prediction and Sequence Annotation

Raw reads of the rare minnow transcriptome were downloaded from GenBank and mapped to the reference genome (unpublished) of rare minnow using the Bowtie software [31]. Potential SNPs were identified by manual methods (eye inspection of the alignments). Sequences 2000 bp in length were captured around the predicted SNP loci from the reference genome for SNP validation.

All captured sequences were aligned to those in the Nt database with the BLASTN program to retrieve genes with the highest sequence similarity to the captured sequences along with their gene function annotations. All sequences were deposited in GenBank under the project PRJNA658832.

All primers were designed using Primer Premier 5.0 software (PREMIER Biosoft International, Palo Alto, CA, USA) to minimize self- and cross-dimerization, and hairpin formation. The primer sequence, annealing temperature (T_m), length, and position are shown in Supplementary Table S1.

2.4. SNP Validation and Genotyping

Six wild rare minnow samples were used to validate the SNPs by SNaPshot methods. A multiplex PCR reaction was performed in a 20 μ L reaction volume comprising 50 ng of DNA template, 7.5 μ L 2 \times PCR master mix, and 2 μ L multiplex primers (10 μ M). The following amplification cycle was used: initial denaturation at 94 $^{\circ}$ C for 3 min, followed by 35 cycles of denaturation at 94 $^{\circ}$ C for 3 min, followed by 35 cycles of denaturation at 94 $^{\circ}$ C for 15 s, annealing at 55 $^{\circ}$ C for 15 s, and extension at 72 $^{\circ}$ C for 30 s, with a final extension at 72 $^{\circ}$ C for 3 min.

Samples were purified post PCR to remove primers and unexhausted dNTPs by ExoI and FastAP enzymes. The reaction mixture comprised 4 μ L PCR product, 0.2 μ L ExoI, 0.8 μ L FastAP, 0.7 μ L ExoI buffer, and 4.7 μ L ddH₂O. The mixture was incubated at 37 $^{\circ}$ C for 15 min, and denatured at 80 $^{\circ}$ C for 15 min. Finally, an extension reaction to complete the PCR amplification was performed and, the reaction mixture comprised 2 μ L PCR product, 1 μ L SNaPshot Mix, and 1 μ L extension primers (10 μ M). The amplification cycle was as follows: denaturation at 96 $^{\circ}$ C for 1 min, followed by 30 cycles of denaturation at 96 $^{\circ}$ C for 10 s, annealing at 52 $^{\circ}$ C for 5 s, and extension at 60 $^{\circ}$ C for 30 s. Sample analysis was performed on an ABI 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Twenty highly variable SNPs were selected for further genotyping of five closed colonies using the above-mentioned method.

2.5. Genetic Analysis

The effective alleles (N_e), observed heterozygosity (H_o), expected heterozygosity (H_e), F -statistics, and genetic distance were calculated using Popgene version 1.32 for each SNP locus. Polymorphic information content (PIC) was calculated using the formula described by Botstein et al. [32].

Hardy–Weinberg equilibrium for each SNP locus was analyzed using the Chi-square test among the six groups. A genetic fingerprint profile was drawn by Heml version 1.0.3.7. To depict divergence among the five closed generations and the wild collection, a phylogenetic tree was constructed by the UPGMA method using Popgene version 1.32 [32] and exported by Mega 5.1 software [33].

3. Results

3.1. SNP Identification and Annotation

Fifty sequences containing potential SNPs were identified using bioinformatic methods. Six wild rare minnow samples were used to validate the 50 sequences by SNaPshot methods. A total of 51 SNPs in 29 sequences were confirmed (Supplementary Table S1). Among the 29 sequences, 14 sequences contained a single SNP, whereas 15 sequences contained two or more SNPs (Supplementary Figure S1). Notably, five SNPs were identified in Seq31. All confirmed SNPs were biallelic except Seq26-2, which had three alleles.

We used BLAST to annotate the SNPs; 33 SNPs were annotated with a few SNPs in important functional genes (Supplementary Table S1).

3.2. Genetic Diversity of Closed Colony and Wild Samples

We selected 20 highly variable SNPs among the 51 SNPs to analyze the genetic diversity among the five closed colonies and the wild group using SNaPshot methods. The five generations of the closed colony were F1, F3, F4, F5, and F10. Among the six groups, all SNPs were moderately or highly polymorphic ($PIC > 0.25$). The range of the effective number of alleles (N_e), mean observed heterozygosity (H_o) and mean expected homozygosity (H_e) in the six groups was 1.8183–1.8745, 0.4333–0.4983, and 0.4445–0.4758, respectively (Table 1).

Table 1. Genetic parameters of six rare minnow populations based on 20 single nucleotide polymorphisms (SNPs).

Loci	Wild			F1			F3		
	N_e	H_o	H_e	N_e	H_o	H_e	N_e	H_o	H_e
Seq3	1.3846	0.3333	0.3030	1.8672	0.5333	0.4723	1.8967	0.2333	0.4808
Seq10-1	1.8000	0.6667	0.4848	1.6423	0.2667	0.3977	1.8000	0.3333	0.4520
Seq11-1	1.3846	0.3333	0.3030	1.3006	0.2667	0.2350	1.3423	0.2333	0.2593
Seq12	1.3846	0.3333	0.3030	1.2195	0.2000	0.1831	1.3006	0.2000	0.2350
Seq13-1	1.6000	0.1667	0.4091	1.5139	0.3667	0.3452	1.5139	0.3000	0.3452
Seq15-1	1.9459	0.5000	0.5303	1.9978	0.3667	0.5079	2.0000	0.4667	0.5085
Seq18-2	1.6000	0.5000	0.4091	1.8672	0.4667	0.4723	1.9912	0.5333	0.5062
Seq26-1	1.9459	0.5000	0.5303	1.8672	0.3333	0.4723	1.8967	0.5000	0.4808
Seq26-2	2.8800	0.5000	0.7121	2.8302	0.8000	0.6576	2.9412	0.6000	0.6712
Seq28	2.0000	0.6667	0.5455	1.7630	0.5667	0.4401	1.4706	0.3333	0.3254
Seq29	1.6000	0.5000	0.4091	1.8672	0.5333	0.4723	1.8000	0.5333	0.4520
Seq30	1.9459	0.1667	0.5303	1.6423	0.3333	0.3977	1.6838	0.3000	0.4130
Seq31-1	1.9459	0.5000	0.5303	1.9459	0.6333	0.4944	1.9802	0.5667	0.5034
Seq31-5	1.9459	0.5000	0.5303	1.9802	0.6333	0.5034	1.9802	0.5667	0.5034
Seq38-1	1.9459	0.1667	0.5303	1.8000	0.5333	0.4520	1.8967	0.5667	0.4808
Seq39-2	2.0000	0.6667	0.5455	1.8967	0.4333	0.4808	1.9651	0.8000	0.4994
Seq42-2	1.9459	0.5000	0.5303	1.9978	0.6333	0.5079	1.9651	0.6000	0.4994
Seq49	1.8000	0.3333	0.4848	1.8349	0.5667	0.4627	1.8967	0.5667	0.4808
Seq51-1	1.8000	0.3333	0.4848	1.9459	0.5000	0.4944	1.8967	0.5000	0.4808
Seq53-2	1.6000	0.5000	0.4091	1.7630	0.5000	0.4401	1.9459	0.7667	0.4944
Mean	1.8228	0.4333	0.4758	1.8271	0.4733	0.4445	1.8582	0.4750	0.4536
St.	0.3295	0.1567	0.1010	0.3203	0.1516	0.1008	0.3373	0.1713	0.0981

Table 1. Cont.

Loci	F4			F5			F10		
	Ne	Ho	He	Ne	Ho	He	Ne	Ho	He
Seq3	1.9978	0.5000	0.5079	1.9912	0.5333	0.5062	1.8824	0.4375	0.4762
Seq10-1	1.7630	0.5000	0.4401	1.3006	0.2000	0.2350	1.7167	0.4062	0.4241
Seq11-1	1.5571	0.4667	0.3638	1.5139	0.3667	0.3452	1.7534	0.3750	0.4365
Seq12	1.4274	0.3667	0.3045	1.3846	0.2667	0.2825	1.4787	0.4062	0.3289
Seq13-1	1.7241	0.4667	0.4271	1.6838	0.3667	0.4130	1.7886	0.4062	0.4479
Seq15-1	1.9459	0.5000	0.4944	1.9978	0.5667	0.5079	1.7167	0.4688	0.4241
Seq18-2	1.9651	0.4667	0.4994	1.8672	0.5333	0.4723	1.9922	0.3125	0.5060
Seq26-1	1.8349	0.2333	0.4627	1.8672	0.5333	0.4723	1.7167	0.4688	0.4241
Seq26-2	2.9364	0.5667	0.6706	2.8986	0.7333	0.6661	2.5222	0.5312	0.6131
Seq28	1.7630	0.3667	0.4401	1.6423	0.4000	0.3977	1.9523	0.4688	0.4955
Seq29	1.7241	0.3333	0.4271	1.6838	0.3667	0.4130	1.9523	0.4688	0.4955
Seq30	1.6838	0.3000	0.4130	1.6838	0.4333	0.4130	1.4787	0.3438	0.3289
Seq31-1	1.9459	0.5000	0.4944	1.9651	0.6000	0.4994	1.8534	0.5312	0.4678
Seq31-5	1.9802	0.5667	0.5034	1.9978	0.5667	0.5079	1.9087	0.5938	0.4836
Seq38-1	1.8000	0.4667	0.4520	1.7630	0.5667	0.4401	1.7534	0.4375	0.4365
Seq39-2	1.8349	0.4333	0.4627	1.8967	0.5667	0.4808	1.8221	0.6250	0.4583
Seq42-2	1.9912	0.7333	0.5062	2.0000	0.6667	0.5085	1.7534	0.4375	0.4365
Seq49	1.8000	0.4667	0.4520	1.7241	0.5333	0.4271	1.7534	0.4375	0.4365
Seq51-1	1.9802	0.5667	0.5034	1.9231	0.5333	0.4881	1.8534	0.4062	0.4678
Seq53-2	1.8349	0.4333	0.4627	1.8349	0.6333	0.4627	1.7167	0.5312	0.4241
Mean	1.8745	0.4617	0.4644	1.8310	0.4983	0.4469	1.8183	0.4547	0.4506
St.	0.2920	0.1094	0.0706	0.3229	0.1348	0.0913	0.2135	0.0782	0.0603

Hardy–Weinberg equilibrium for each SNP locus was analyzed using the Chi-square test among the six groups. The mean range of p -value of Hardy–Weinberg equilibrium in the six groups was 0.3716–0.5415 (Table 2), indicating random mating and no-evidence for inbreeding. The frequency of all 20 SNP loci in the wild and the F1 generation conformed to the Hardy–Weinberg equilibrium. There was one locus each in the F5 and F10 generations, two loci in the F4 generation, and three loci in the F3 generation that deviated from Hardy–Weinberg equilibrium (Table 2).

Table 2. Hardy–Weinberg equilibrium with *p*-value among six rare minnow populations based on 20 SNPs. Numbers in bold identify significant deviations from Hardy–Weinberg equilibrium.

Loci	Wild	F1	F3	F4	F5	F10
Seq3	0.7389	0.4705	0.0041	0.9309	0.7653	0.6398
Seq10-1	0.3006	0.0637	0.1419	0.4459	0.3863	0.8076
Seq11-1	0.7389	0.4339	0.5632	0.1103	0.7246	0.4155
Seq12	0.7389	0.5814	0.3863	0.2442	0.7481	0.1685
Seq13-1	0.0852	0.7246	0.4582	0.6036	0.5287	0.5913
Seq15-1	0.8774	0.1213	0.6469	0.9492	0.5193	0.5426
Seq18-2	0.5186	0.9467	0.7653	0.7146	0.4705	0.0280
Seq26-1	0.8774	0.1002	0.8235	0.0056	0.4705	0.5426
Seq26-2	0.3165	0.1124	0.1419	0.1725	0.8575	0.5707
Seq28	0.5510	0.1072	0.8901	0.3499	0.9745	0.7559
Seq29	0.5186	0.4705	0.3138	0.2181	0.5287	0.7559
Seq30	0.0641	0.3623	0.1244	0.1244	0.7821	0.7911
Seq31-1	0.8774	0.1169	0.4836	0.9492	0.2617	0.4340
Seq31-5	0.8774	0.1503	0.4836	0.4836	0.5193	0.1899
Seq38-1	0.0641	0.3138	0.3187	0.8557	0.1072	0.9895
Seq39-2	0.5510	0.5816	0.0008	0.7226	0.3187	0.0360
Seq42-2	0.8774	0.1689	0.2617	0.0124	0.0831	0.9895
Seq49	0.3884	0.2091	0.3187	0.8557	0.1631	0.9895
Seq51-1	0.3884	0.9492	0.8235	0.4836	0.6055	0.4485
Seq53-2	0.5186	0.4459	0.0021	0.7226	0.0393	0.1439
mean	0.5434	0.3715	0.3976	0.4977	0.4927	0.5415
Std	0.2741	0.2701	0.2794	0.3203	0.2632	0.3014

3.3. Genetic Structure Analysis of Closed Colony

Distribution analysis of the dominant genotype showed maintained stability between the five generations for five SNP loci including Seq11-1 (GG), Seq12 (GG), Seq30 (CC), Seq31-1 (CT), and Seq31-5 (AG) (Figure 1c, 1d, 1m, 1n). The least frequent genotype distribution analysis showed maintained stability among the five closed colonies for fifteen SNP loci including Seq10-1 (GG), Seq11-1 (CC), Seq12 (AA), Seq13-1 (GG), Seq26-1 (TT), Seq28 (CC), Seq29 (TT), Seq30 (TT), Seq31-1 (CC), Seq31-5 (AA), Seq38-1 (TT), Seq39-2 (TT), Seq49 (AA), Seq51-1 (GG), and Seq53-2 (CC) (Figure 1). The frequency of dominant and least allele types between the F5 and F10 generations was constant (Figure 1).

The range of mean N_e , H_o , and H_e in F1, F3, F4, F5, and F10 was 1.8183–1.8745, 0.4547–0.4983, and 0.4445–0.4644, respectively (Table 1). The F4 presented the highest mean N_e (1.8745) and mean H_e (0.4644), whereas the F5 generation presented the highest mean H_o (0.4983). There were no significant differences among the five closed colonies for genetic parameters of N_e , H_o , and H_e ($p > 0.05$), in the one-way ANOVA (Figure 2), indicating that heterozygosity in IHB was well maintained.

F -statistics (F_{IS} , F_{IT} , and F_{ST}) analysis of the five rare minnow closed colonies demonstrated positive F_{IS} values for nine SNP loci, and positive F_{IT} values for seven SNP loci (Table 3). The mean F_{IS} and F_{IT} values were -0.2659 and -0.0631 , respectively, with negative values indicating low inbreeding within a closed colony. The mean F_{ST} value of the five closed colonies was 0.0146 (Table 3), whereas F_{ST} between generations ranged from 0.0032 to 0.0163 (Table 4), and the mean F_{ST} was 0.0146, the F_{ST} values indicated low genetic differentiation between the five successive generations.

Table 3. F -statistics of 20 SNPs loci among six rare minnow populations.

Loci	F_{IS}	F_{IT}	F_{ST}
Seq3	0.0689	0.1021	0.0356
Seq10-1	0.1099	0.1331	0.026
Seq11-1	−0.0591	−0.0327	0.0249
Seq12	−0.0972	−0.0861	0.0101
Seq13-1	0.0204	0.0305	0.0103
Seq15-1	0.014	0.0514	0.0379
Seq18-2	0.0428	0.075	0.0337
Seq26-1	0.0903	0.0939	0.0039
Seq26-2	−0.0021	0.0082	0.0103
Seq28	−0.0344	−0.0086	0.025
Seq29	−0.0057	0.0053	0.0109
Seq30	0.1152	0.1196	0.005
Seq31-1	−0.1705	−0.166	0.0038
Seq31-5	−0.1896	−0.1853	0.0036
Seq38-1	−0.1559	−0.1526	0.0029
Seq39-2	−0.22	−0.2148	0.0043
Seq42-2	−0.27	−0.2366	0.0263
Seq49	−0.157	−0.1526	0.0038
Seq51-1	−0.0467	−0.0427	0.0039
Seq53-2	−0.2752	−0.2659	0.0073
Mean	−0.0631	−0.0476	0.0146

Table 4. Matrix of F_{ST} and genetic distance among generations.

Generation	F1	F3	F4	F5	F10
F1	****	0.0034	0.0058	0.0077	0.015
F3	0.0054	****	0.0055	0.0066	0.0163
F4	0.0025	0.0091	****	0.0032	0.0126
F5	0.0122	0.0106	0.0052	****	0.0153
F10	0.0242	0.027	0.0211	0.0249	****

Note: F_{ST} (above diagonal) and genetic distance (below diagonal); **** means null value.

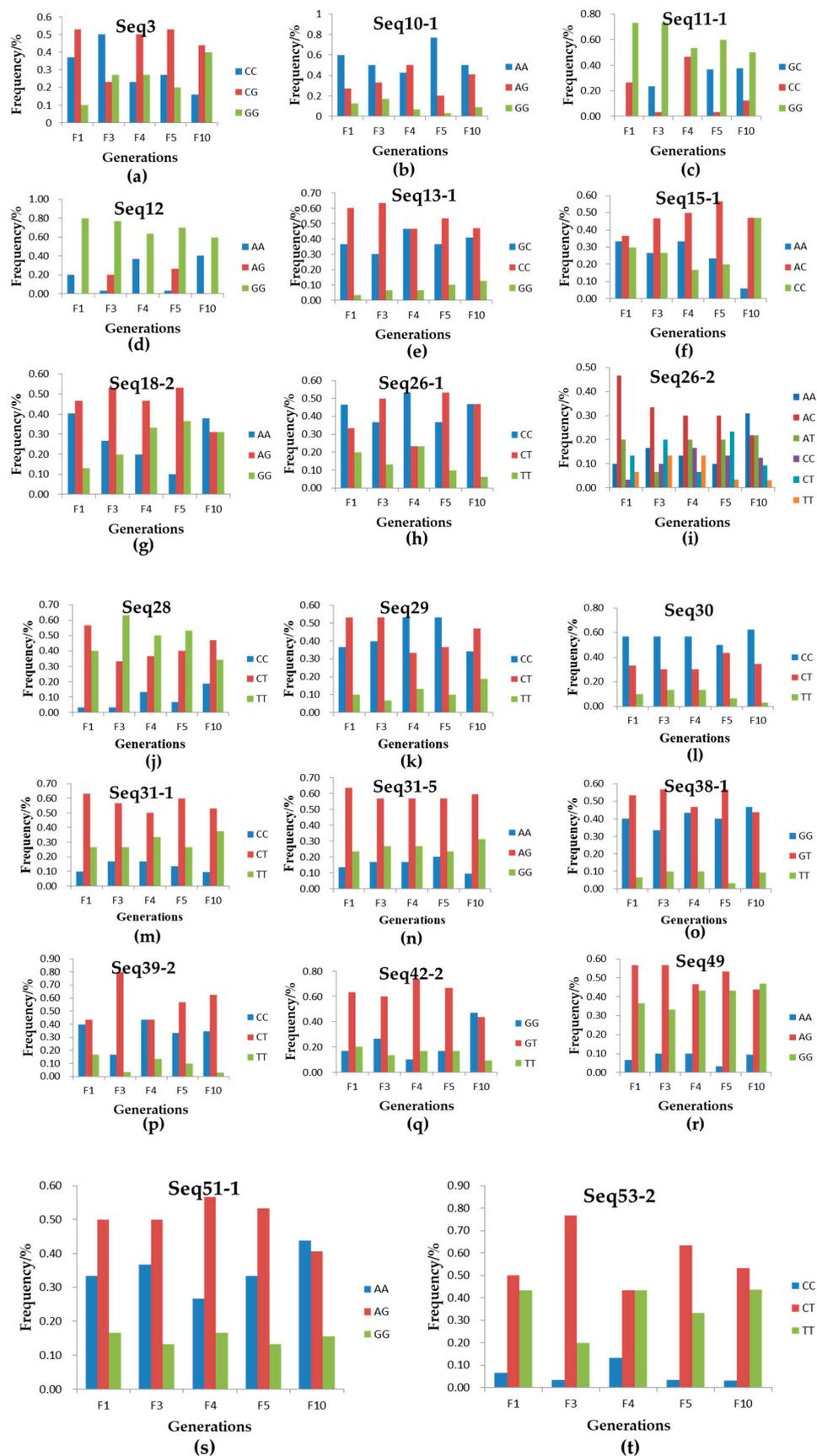


Figure 1. Genotype frequency distributions of 20 SNPs loci in the five closed colonies. (a)–(t) Individual SNP genotype frequencies among the five generations.

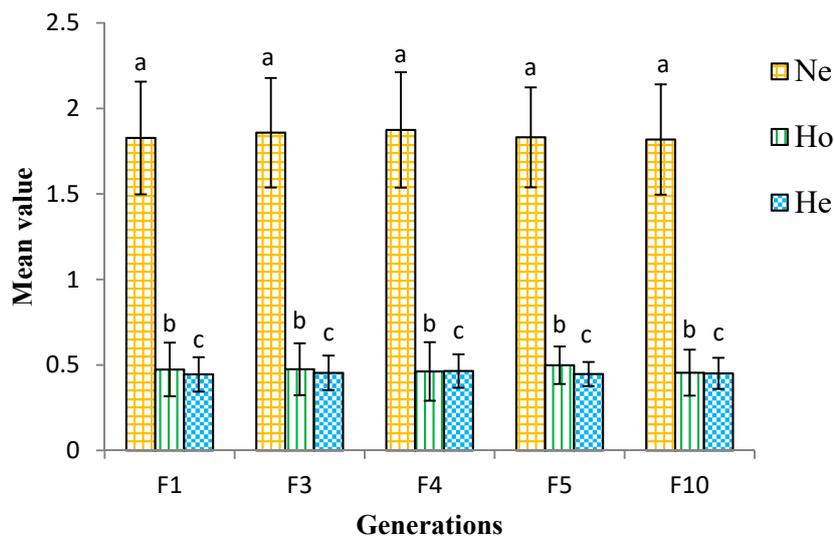


Figure 2. Comparison of genetic parameters among five populations. Means of exposure sharing a common letter are not significantly different at $p > 0.05$ as assessed using a one-way ANOVA followed by Dunnett’s test.

The UPGMA phylogenetic tree of the five closed generations and wild fish, based on genetic distances (Table 4), suggested the distinction of the F10 (Figure 3). With breeding generations in closed colonies, the genetic distance between the older and newer generations tended to increase.

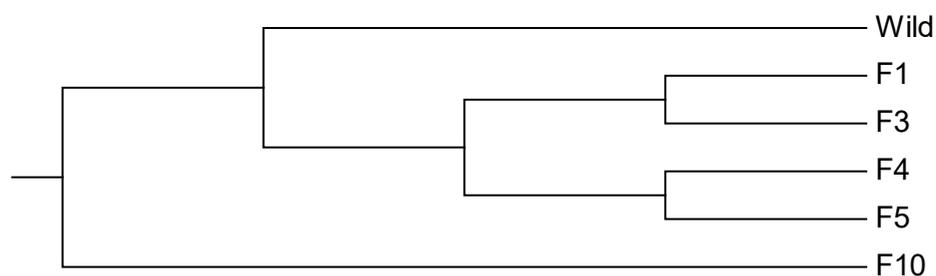


Figure 3. Phylogenetic tree of six groups constructed using the UPGMA clustering method based on Nei’s genetic distances (Table 4) for the 20 SNPs loci.

With the allele information of the 20 SNP loci, the Heml software was used to draw the genotype distribution profile heat map of the five IHB closed colonies. Clustering analysis showed the grouping of the 4 SNPs as an independent branch separated from the other 16 SNPs (Figure 4).

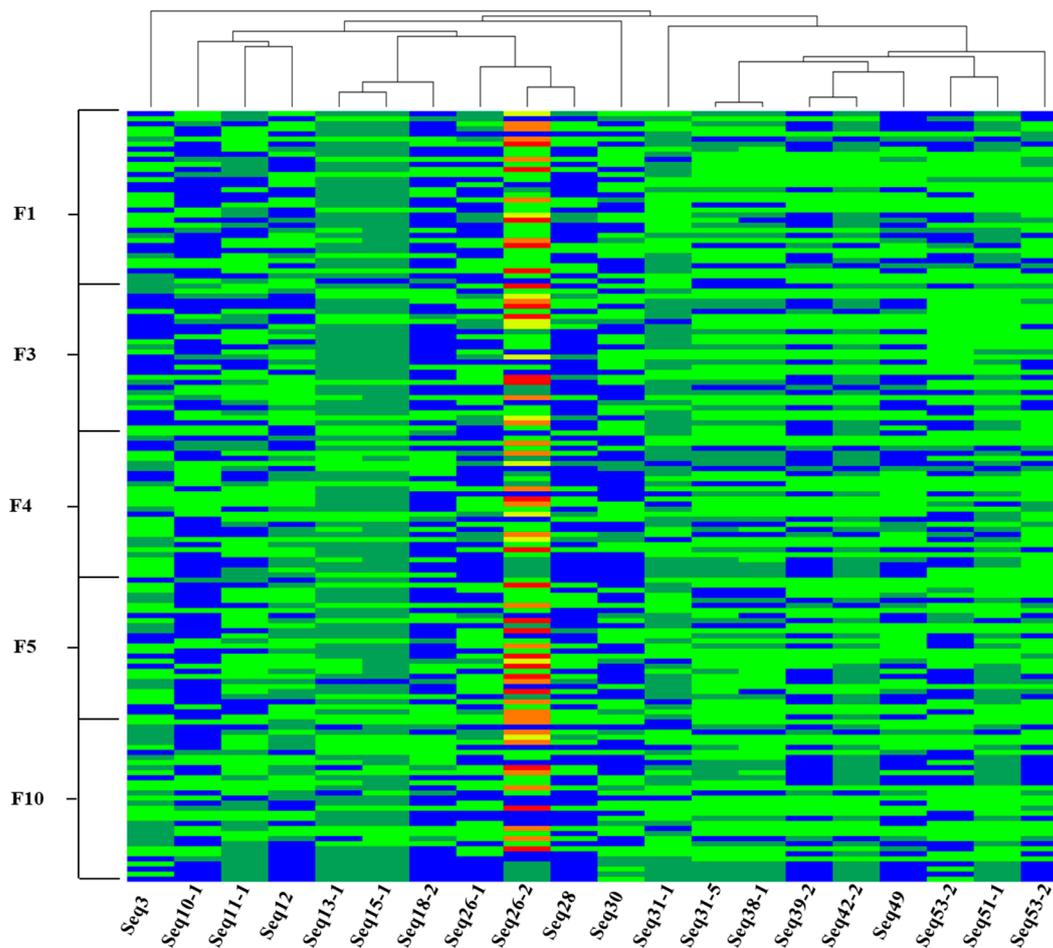


Figure 4. Heat map of the genotype distribution profile of the five rare minnow closed colonies.

3.4. Differences in the Results of the SNP and SSR Marker Analyses in the Same Samples

Previously, we analyzed the genetic structure of the F1–F5 IHB generations using the SSR markers (12 loci) [34]. Here, we compared the results of SNP and SSR marker analysis in the same samples (Table 5) and found consistent results between the two methods (F1, F3, F4, and F5). There were no significant differences in the genetic parameters of N_e , H_o , and H_e among the four closed colonies ($p > 0.05$). The genetic distance analysis showed similar results, and with the generation of IHB, the genetic distance between older and newer generations gradually increased.

Table 5. Comparison of genetic indexes determined using SNP and SSR markers.

Loci	N_e		H_o		H_e	
	SNP	SSR [34]	SNP	SSR [34]	SNP	SSR [34]
F1	1.8271	3.40	0.4733	0.5651	0.4445	0.5733
F3	1.8582	3.20	0.4750	0.5267	0.4536	0.5647
F4	1.8745	3.30	0.4617	0.5578	0.4644	0.5689
F5	1.8310	3.20	0.4983	0.5164	0.4469	0.5607

4. Discussion

4.1. Characteristics of SNP Markers in Rare Minnow

Compared with traditional model animals, basic genomic information of newly developed model organisms is limited. As a newly emerging laboratory fish, the absence of genome assembly of the

rare minnow has notably hampered the development of genetic markers [35–37]. Moreover, there are no SNP markers for the rare minnow. To the best of our knowledge, the present study is the first to identify 51 SNP markers in 29 rare minnow genome sequences, with about 50% sequences containing two or more SNP loci, and five SNPs identified in Seq31, reflecting an abundance of SNPs in the rare minnow genome. Most SNPs in the vertebrate genome are biallelic; however, due to both transition and transversion mutations at CpG dinucleotide [38], triallelic SNPs are observed as well. A triallelic SNP (A/C/T) in locus of Seq26-2 was identified and triallelic SNP was observed in other fish [39]. BLAST could annotate 33 SNPs, and the remaining SNPs may be located in an intron or regulatory region. The annotated SNPs represent some critical genes including forkhead box P2, MyoD family inhibitor domain-containing protein, and limb development membrane protein. SNPs located in coding regions may provide valuable insights for further functional gene research in the rare minnow.

4.2. Relationship between Heterozygosity of Genetic Markers and Population History

According to the classic definitions of laboratory animals' genetic background, the maintenance of limited genetic diversity is an essential requirement of a closed colony [40]. Heterozygosity has been introduced to genetic diversity research as a core indicator of genetic diversity [34,41,42]. The value of heterozygosity exhibits differences due to the use of different methods, such as genetic analysis using SNPs and SSRs between wild and seven cultured samples of pacific oyster (*Crassostrea gigas*) demonstrated lower mean heterozygosity using SNPs than with SSRs [43]. A similar result was obtained in wild pacu (*Piaractus mesopotamicus*), wherein the mean heterozygosity determined with SNPs and SSRs was 0.423 and 0.612, respectively [44]. In this study, we analyzed successive generations of IHB using SNPs and the range of heterozygosity was 0.4445–0.4983, which was lower than that reported in a previous study using SSRs [34]. The population heterozygosity was closely related to the type of genetic marker. For the inherent differences between SNPs and SSRs, SNPs were often exhibited diallelic, whereas SSRs may contain dozens of alleles. The SSR mutation rate per locus is higher than that of an SNP (10^{-6} to 10^{-2} compared with 10^{-9}). Hence, the stability of SNP is higher than that of SSR during DNA replication [45], leading to lower heterozygosity of SNP markers than SSR markers. Furthermore, discrepancies can also be observed using the same method, likely resulting from the selection of different genetic markers. The location of the marker in genome could influence the stability of the locus to some extent, as the mutation rate of a locus may decrease because of the natural selection pressure [46], and the representativeness of markers at different chromosomes should be considered. Therefore, it is necessary to consider the marker type and marker representativeness before evaluation of genetic variability.

The background loss of heterozygosity in the natural population should be another important consideration for the low detection value of genetic heterozygosity. In our previous studies, we found that the genetic diversity in a topotype population of rare minnow (between 1997 and 2006) was moderate [42], with similar results in nine wild populations in the upper Yangtze River [47]. The habitat of rare minnow is mainly small water systems such as paddy fields, ditches, and loblollies [26,27]. All known habitats of the rare minnow are a dozen to a hundred miles away from one another, isolated or separated by large rivers [42]. The narrow habitat environment may be the cause of moderate diversity. In our study, the founder population of IHB was primarily derived from a topotype population as mentioned above, and this may be the reason for the slightly flat heterozygosity in IHB.

As heterozygosity may be an excellent indicator of genetic variability evaluation in a closed colony, it might be challenging to reach an agreement about the criterion of heterozygosity among different methods or species. For instance, in genetic variability testing of laboratory animals, based on SSR markers, the threshold of heterozygosity is generally recognized to be between 0.5 and 0.7 [41]. However, the criterion of heterozygosity in SSR analysis may not apply to the SNP method. Considering the requirements of closed colonies, maintaining stable heterozygosity between generations and establishing a quality control chart may be good indicators for the SNP methods.

In our study, no significant differences in H_o and H_e were observed between the five closed colonies, indicating homogeneity in the genetic diversity among IHBs over time.

4.3. Stability Maintenance of the Genetic Structure in a Closed Colony

In order to understand the genetic structure of a closed colony more comprehensively, there is a need for more indicators other than heterozygosity, such as population genetic equilibrium, F -statistics (F_{ST}), and genetic distance. Population genetic equilibrium is an important indicator to evaluate the genetic structure, and is closely related to gene mutation, genetic drift, and natural selection of population [48]. In some studies [49] and national standards [2], the genetic equilibrium has been determined for the evaluation of the genetic variability of closed colonies. Ideally, the genetic structure of a well-managed closed colony may be equalized between generations. In this study, we found that all five closed colony generations conformed to the equilibrium based on the mean Hardy–Weinberg equilibrium test p -value. However, one locus in the F10 generation deviated from Hardy–Weinberg equilibrium, which may be related to the position of this SNP in the genome, or alternatively, the effect of domestication of this generation and representativeness of samples.

F -statistics (F_{ST}) was used to indicate differentiation among successive generations within the closed colony, with values ranging between 0–0.05, 0.05–0.15, and 0.15–0.25, indicating “little,” “moderate,” and “great” genetic differentiation, respectively, as stated by Wright et al. [50]. In our study, the F_{ST} value of 0.0146 indicated that genetic differentiation among the five generations was negligible and that it may be associated with the breeding strategy of IHB. The foundation stock of IHB was bred by strictly following a maximum non-inbred method [2].

In a well-managed closed colony, there should be minor differences in the genetic distance between successive generations. However, because all mating events in a closed colony occur among colony members, some weak alleles associated with reproductive capacity might be lost gradually over several generations. Thus, there might be an accumulation of low genetic differentiation with continuous passage in a closed colony. In our study, although the key genetic parameters showed no significant differences within the five groups, we observed a gradual increase in genetic distance among these five closed colony generations (F1 to F10), between early and later generations. It is difficult to avoid the accumulation of a few genetic differentiations during passage in a closed colony. For a qualified closed colony, it is necessary to maintain a stable genetic distance between adjacent generations.

Meanwhile, reinforcing breeding management strategies for the foundation stock is also indispensable to the maintenance of the stability of the genetic background, by avoiding any selection pressure on the parent fish for as long as possible, maintaining an equal probability of transferring an allele in the whole population, and decreasing the probability of genetic drift among generations [51]. Meanwhile, according to the report of Jackson Laboratory, the embryo freeze method can be used to potentially decrease the passage number of closed colonies [52].

In summary, maintaining stable genetic diversity and genetic structure may be the primary consideration for a qualified closed colony. It is necessary to select appropriate evaluation indicators, such as stability of heterozygosity, genetic equilibrium, F_{ST} , and genetic distance, and also consider the effect factors and sphere of application of each index, when conducting a genetic variability evaluation of a closed colony.

5. Conclusions

In the present study, we developed SNP markers for the rare minnow for the first time. We also conducted genetic diversity and structure analyses in successive generations of the IHB strain, establishing a genetic variability monitoring method of rare minnow closed colonies using SNPs. Our study revealed stable genetic structure in successive generations, demonstrating the success of the reproductive strategies used for IHB. The results of this study could help establish genetic variability control standards for the rare minnow, maintain resources for wild population restoration, and provide a reference for research on other laboratory fish.

Supplementary Materials: The following are available online at <http://www.mdpi.com/1424-2818/12/12/483/s1>, Figure S1: Frequency distribution of 51 SNPs in 29 sequences; Table S1: Characterization of 51 SNP markers in the rare minnow.

Author Contributions: Conceptualization, J.W.; methodology, L.C. and M.H.; validation, C.X.; investigation, Z.X.; writing—original draft preparation, L.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Hubei Key Technology R&D Program (grant number 2015BCE098).

Acknowledgments: We thank M.H. Wu, D. Yu, L.X. Su, N. Qiu, and F.T. Zhang for their assistance in sample collection and English language editing.

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

References

- Hau, J. *Handbook of Laboratory Animal Science*, 3rd ed.; Essential Principles and Practices; CRC Press: Boca Raton, FL, USA, 2013; Volume I.
- National Technical Committee 281 on Laboratory Animal of Standardization Administration of China. *Laboratory Animal-Genetic Quality Control of Mammalian Laboratory Animals, GB 14923-2010*; Standards Press of China: Beijing, China, 2010.
- Leo, S.S.T.; Millien, V. Microsatellite markers reveal low frequency of natural hybridization between the white-footed mouse (*Peromyscus leucopus*) and deer mouse (*Peromyscus maniculatus*) in southern Quebec, Canada. *Genome* **2017**, *60*, 454–463. [[CrossRef](#)] [[PubMed](#)]
- Jo, Y.S.; Kim, H.N.; Baccus, J.T.; Jung, J. Genetic differentiation of the Korean striped field mouse, *Apodemus agrarius* (Muridae, Rodentia), based on microsatellite polymorphism. *Mammalia* **2017**, *81*, 297–307. [[CrossRef](#)]
- Lai, F.Y.; Ding, S.T.; Tu, P.A.; Chen, R.S.; Lin, D.Y.; Lin, E.C.; Wang, P.H. Population structure and phylogenetic analysis of laboratory rabbits in Taiwan based on microsatellite markers. *World Rabbit Sci.* **2018**, *26*, 57–70. [[CrossRef](#)]
- Vignal, A.; Milan, D.; SanCristobal, M.; Eggen, A. A review on SNP and other types of molecular markers and their use in animal genetics. *Genet. Sel. Evol.* **2002**, *34*, 275–305. [[CrossRef](#)] [[PubMed](#)]
- Bianchi, D.; Brancadoro, L.; De Lorenzis, G. Genetic diversity and population structure in a *Vitis spp.* Core collection investigated by SNP markers. *Diversity* **2020**, *12*, 103. [[CrossRef](#)]
- Plomion, C.; Bartholome, J.; Lesur, I.; Boury, C.; Rodriguez-Quilon, I.; Lagraulet, H.; Ehrenmann, F.; Bouffier, L.; Gion, J.M.; Grivet, D.; et al. High-density SNP assay development for genetic analysis in maritime pine (*Pinus pinaster*). *Mol. Ecol. Resour.* **2016**, *16*, 574–587. [[CrossRef](#)]
- Johnston, H.R.; Hu, Y.J.; Gao, J.; O'Connor, T.D.; Abecasis, G.R.; Wojcik, G.L.; Gignoux, C.R.; Gourraud, P.A.; Lizee, A.; Hansen, M.; et al. Identifying tagging SNPs for African specific genetic variation from the African Diaspora Genome. *Sci. Rep.* **2017**, *7*, 46398. [[CrossRef](#)]
- Romanowska, J.; Haaland, O.A.; Jugessur, A.; Gjerdevik, M.; Xu, Z.; Taylor, J.; Wilcox, A.J.; Jonassen, I.; Lie, R.T.; Gjessing, H.K. Gene-methylation interactions: Discovering region-wise DNA methylation levels that modify SNP-associated disease risk. *Clin. Epigenet.* **2020**, *12*, 109. [[CrossRef](#)]
- Petkov, P.M.; Ding, Y.; Cassell, M.A.; Zhang, W.; Wagner, G.; Sargent, E.E.; Asquith, S.; Crew, V.; Johnson, K.A.; Robinson, P.; et al. An efficient SNP system for mouse genome scanning and elucidating strain relationships. *Genome Res.* **2004**, *14*, 1806–1811. [[CrossRef](#)]
- Sun, F.Y.; Wang, Y.J.; Yang, W.F.; Che, L.P.; Sheng-Ming, L.U.; Wang, X.K.J.L.A.S. Advances of microsatellite and single nucleotide polymorphism analysis for genetic monitoring in mouse and rat. *Lab. Anim. Sci.* **2014**, *31*, 60–65.
- Churchill, G.A.; Gatti, D.M.; Munger, S.C.; Svenson, K.L. The diversity outbred mouse population. *Mamm. Genome* **2012**, *23*, 713–718. [[CrossRef](#)] [[PubMed](#)]
- Yalcin, B.; Nicod, J.; Bhomra, A.; Davidson, S.; Cleak, J.; Farinelli, L.; Osteras, M.; Whitley, A.; Yuan, W.; Gan, X.; et al. Commercially available outbred mice for genome-wide association studies. *PLoS Genet.* **2010**, *6*, e1001085. [[CrossRef](#)] [[PubMed](#)]

15. Parker, C.C.; Gopalakrishnan, S.; Carbonetto, P.; Gonzales, N.M.; Leung, E.; Park, Y.J.; Aryee, E.; Davis, J.; Blizard, D.A.; Ackert-Bicknell, C.L.; et al. Genome-wide association study of behavioral, physiological and gene expression traits in outbred CFW mice. *Nat. Genet.* **2016**, *48*, 919–926. [[CrossRef](#)] [[PubMed](#)]
16. Aldinger, K.A.; Sokoloff, G.; Rosenberg, D.M.; Palmer, A.A.; Millen, K.J. Genetic variation and population substructure in outbred CD-1 mice: Implications for genome-wide association studies. *PLoS ONE* **2009**, *4*, e4729. [[CrossRef](#)]
17. Ostrander, G.K.; Hopkins, J. *The Laboratory Fish*; Academic Press: Pittsburgh, PA, USA, 2000.
18. Peterson, R.T.; Shaw, S.Y.; Peterson, T.A.; Milan, D.J.; Zhong, T.P.; Schreiber, S.L.; MacRae, C.A.; Fishman, M.C. Chemical suppression of a genetic mutation in a zebrafish model of aortic coarctation. *Nat. Biotechnol.* **2004**, *22*, 595–599. [[CrossRef](#)]
19. Dang, Z.; van der Ven, L.T.M.; Kienhuis, A.S. Fish embryo toxicity test, threshold approach, and moribund as approaches to implement 3R principles to the acute fish toxicity test. *Chemosphere* **2017**, *186*, 677–685. [[CrossRef](#)]
20. Shinya, M.; Sakai, N. Generation of Highly Homogeneous Strains of Zebrafish Through Full Sib-Pair Mating. *Genes Genomes Genet.* **2011**, *1*, 377–386. [[CrossRef](#)]
21. Qiu, N.; Su, L.; Wu, B.; Hou, M.; Xu, C.; Wang, J. Chemicals affect color preference in rare minnow (*Gobiocypris rarus*). *Environ. Sci. Pollut. Res. Int.* **2020**, *27*, 23206–23214. [[CrossRef](#)]
22. Kinoshita, M.; Murata, K.; Naruse, K.; Tanaka, M. *Medaka: Biology, Management, and Experimental Protocols*; John Wiley & Sons: Hoboken, NJ, USA, 2009.
23. Morizot, D.C.; Wright, D.A.; Siciliano, M.J. Three linked enzyme loci in fishes: Implications in the evolution of vertebrate chromosomes. *Genetics* **1977**, *86*, 645–656.
24. Shikano, T.; Taniguchi, N. Using microsatellite and RAPD markers to estimate the amount of heterosis in various strain combinations in the guppy (*Poecilia reticulata*) as a fish model. *Aquaculture* **2002**, *204*, 271–281. [[CrossRef](#)]
25. Cai, L.; Li, J.; Yu, L.; Wei, Y.; Miao, Z.; Chen, M.; Huang, R. Characterization of transcriptional responses mediated by benzo[a]pyrene stress in a new marine fish model of goby, *Mugilogobius chulae*. *Genes Genom.* **2019**, *41*, 113–123. [[CrossRef](#)] [[PubMed](#)]
26. Su, L.X.; Luo, S.; Qiu, N.; Xiong, X.Q.; Wang, J.W. The reproductive strategy of the rare minnow (*Gobiocypris rarus*) in response to starvation stress. *Zool. Stud.* **2020**, *59*, 1–12.
27. Wang, J.W.; Cao, W.X. *Gobiocypris rarus* as a Chinese native model organism: History and current situation. *Asian J. Ecotoxicol.* **2017**, *12*, 20–33.
28. Gu, D.E.; Yu, X.Y.; Wang, J.W. Monitoring of the growth and reproductive performance of a rare closed colony of *Gobiocypris rarus* Ihb: IHB. *Acta. Lab. Anim. Sci. Sin.* **2014**, *22*, 71–75.
29. Gu, D.E.; Wang, J.W. Application of microsatellite marker in genetic monitoring on the foundation of a closed colony of *Gobiocypris rarus*. *Acta Hydrobiol. Sin.* **2012**, *36*, 197–204.
30. Hu, C.T.; Yan, J.W.; Chen, F.; Zhang, Q.X.; Wang, H.D.; Yin, C.Y.; Fan, H.T.; Hu, L.L.; Shen, C.M.; Meng, H.T.; et al. Genetic analysis of 15 mtDNA SNP loci in Chinese Yi ethnic group using SNaPshot minisequencing. *Gene* **2016**, *576*, 105–108. [[CrossRef](#)]
31. Langmead, B. Aligning Short Sequencing Reads with Bowtie. *Curr. Protoc. Bioinform.* **2010**, *32*, 11.7.1–11.7.4. [[CrossRef](#)]
32. Botstein, D.; White, R.L.; Skolnick, M.; Davis, R.W. Construction of a genetic-linkage map in man using restriction fragment length polymorphisms. *Am. J. Hum. Genet.* **1980**, *32*, 314–331.
33. Tamura, K.; Peterson, D.; Peterson, N.; Stecher, G.; Nei, M.; Kumar, S.J.M.B. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **2011**, *28*, 2731–2739. [[CrossRef](#)]
34. Li, H.; Wang, C.; Wang, J. Genetic structure of IHB rare minnow (*Gobiocypris rarus*). *J. Hydroecol.* **2018**, *39*, 83–90.
35. Goldstein, B.; King, N. The future of cell biology: Emerging model organisms. *Trends Cell Biol.* **2016**, *26*, 818–824. [[CrossRef](#)] [[PubMed](#)]
36. Cook, C.E.; Chenevert, J.; Larsson, T.A.; Arendt, D.; Houlston, E.; Lenart, P. Old knowledge and new technologies allow rapid development of model organisms. *Mol. Biol. Cell.* **2016**, *27*, 882–887. [[CrossRef](#)] [[PubMed](#)]
37. Jenner, R.A.; Wills, M.A. The choice of model organisms in evo-devo. *Nat. Rev. Genet.* **2007**, *8*, 311–319. [[CrossRef](#)] [[PubMed](#)]

38. Hodgkinson, A.; Eyre-Walker, A. Human triallelic sites: Evidence for a new mutational mechanism? *Genetics* **2010**, *184*, 233–376. [[CrossRef](#)]
39. Roesti, M.; Kueng, B.; Moser, D.; Berner, D. The genomics of ecological vicariance in threespine stickleback fish. *Nat. Commun.* **2015**, *6*, 8767. [[CrossRef](#)]
40. Chia, R.; Achilli, F.; Festing, M.F.W.; Fisher, E.M.C. The origins and uses of mouse outbred stocks. *Nat. Genet.* **2005**, *37*, 1181–1186. [[CrossRef](#)]
41. Liu, X.; Yu, X.; Xu, Y.; Du, X.; Huo, X.; Li, C.; Lv, J.; Guo, M.; Lu, J.; Chen, Z. Development of an effective microsatellite marker system to determine the genetic structure of *Meriones meridianus* populations. *Exp. Anim.* **2020**, *69*, 224–232. [[CrossRef](#)]
42. He, Y.; Wang, J. Temporal variation in genetic structure of the Chinese Rare Minnow (*Gobiocypris rarus*) in its type locality revealed by microsatellite markers. *Biochem. Genet.* **2010**, *48*, 312–325. [[CrossRef](#)]
43. Kong, L.; Bai, J.; Li, Q. Comparative assessment of genomic SSR, EST-SSR and EST-SNP markers for evaluation of the genetic diversity of wild and cultured Pacific oyster, *Crassostrea gigas* Thunberg. *Aquaculture* **2014**, *420*, S85–S91. [[CrossRef](#)]
44. Antonio Mastrochirico-Filho, V.; del Pazo, F.; Elissa Hata, M.; Vanina Villanova, G.; Foresti, F.; Vera, M.; Martinez, P.; Porto-Foresti, F.; Teruo Hashimoto, D. Assessing genetic diversity for a pre-breeding program in *Piaractus mesopotamicus* by SNPs and SSRs. *Genes* **2019**, *10*, 668. [[CrossRef](#)]
45. Eckert, K.A.; Hile, S.E. Every microsatellite is different: Intrinsic DNA features dictate mutagenesis of common microsatellites present in the human genome. *Mol. Carcinog.* **2009**, *48*, 379–388. [[CrossRef](#)] [[PubMed](#)]
46. Lynch, M.; Ackerman, M.S.; Gout, J.F.; Long, H.; Sung, W.; Thomas, W.K.; Foster, P.L. Genetic drift, selection and the evolution of the mutation rate. *Nat. Rev. Genet.* **2016**, *17*, 704–714. [[CrossRef](#)] [[PubMed](#)]
47. He, Y.F.; Wang, J.W.; Blanchet, S.; Lek, S. Genetic structure of an endangered endemic fish (*Gobiocypris rarus*) in the upper Yangtze River. *Biochem. Syst. Ecol.* **2012**, *43*, 214–225. [[CrossRef](#)]
48. Mitchell-Olds, T.; Willis, J.H.; Goldstein, D.B. Which evolutionary processes influence natural genetic variation for phenotypic traits? *Nat. Rev. Genet.* **2007**, *8*, 845–856. [[CrossRef](#)] [[PubMed](#)]
49. Valdez, L.; Quiroga-Carmona, M.; D’Elia, G. Genetic variation of the Chilean endemic long-haired mouse *Abrothrix longipilis* (Rodentia, Supramyomorpha, Cricetidae) in a geographical and environmental context. *PeerJ* **2020**, *8*, e9517. [[CrossRef](#)]
50. Wright, S. *Evolution and the Genetics of Populations: A Treatise in Four Volumes: Vol. 4: Variability within and Among Natural Populations*; University of Chicago Press: Chicago, IL, USA, 1978.
51. Khersonsky, S.M.; Jung, D.W.; Kang, T.W.; Walsh, D.P.; Moon, H.S.; Jo, H.; Jacobson, E.M.; Shetty, V.; Neubert, T.A.; Chang, Y.T. Facilitated forward chemical genetics using a tagged triazine library and zebrafish embryo screening. *J. Am. Chem. Soc.* **2003**, *125*, 11804–11805. [[CrossRef](#)] [[PubMed](#)]
52. Simianer, H.; Koehn, F. Genetic management of the Gottingen Minipig population. *J. Pharmacol. Toxicol. Methods* **2010**, *62*, 221–226. [[CrossRef](#)]

Publisher’s Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>).