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Establishment and Application of Microsatellite Multiplex PCR System for *Cheilinus undulatus*

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Abstract: *Cheilinus undulatus* is a valuable seawater economic fish with tender meat, fresh taste, and high nutritional value; however, its population is rapidly declining because of massive fishing and habitat destruction. Assessing changes in genetic diversity and inbreeding levels is a very valuable monitoring tool, and multiplex PCR has the advantages of being time-efficient and economical. Here, we selected 12 pairs of polymorphic microsatellite loci, developed two multiplex PCR amplification systems based on these microsatellites, and used them to examine 30 *C. undulatus* specimens. The number of alleles (Na) for the 12 microsatellite markers ranged from 2 to 8. The effective allele number (Ne) ranged from 1.724 to 4.592. The expected heterozygosity (He) and observed heterozygosity (Ho) ranged from 0.420 to 0.782 and 0.100 to 0.900, respectively. The polymorphic information content (PIC) ranged from 0.422 to 0.746, with a mean value of 0.557. 5 loci deviated from Hardy-Weinberg equilibrium (HWE, $p < 0.05$ after Bonferroni correction). The multiplex PCR amplification system established in this study provides a basis for germplasm identification, genetic diversity analysis, and assessment of the effects of accretion and release of *C. undulatus*.

Keywords: *Cheilinus undulatus*; microsatellite; multiplex PCR; genetic diversity

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1. Introduction

Cheilinus undulatus, known as Maori, Napoleon, humphead wrasse, and so-mei [1], belongs to the order Perciformes [2]. The species is found in reefs and nearshore habitats with seagrass beds and mangroves distributed in tropical waters of the Pacific and Indian Oceans [3]. Its abundance is usually very low, and it feeds on mollusks, small fish, sea urchins, and crustaceans [4]. *C. undulatus* is one of the most valuable and expensive fish species in coral reefs [5], and the large coral triangle is the main distribution area [6]. Due to the heavy exploitation of the live reef fish trade (LRFT), it is classified as "vulnerable" in the IUCN 1996 Red Data Book [1]. International regulations treat *C. undulatus* as a wild fish that can be traded within a limited quota [7]. Human activities are a major cause of biodiversity decline, and marine animal extinctions began to accelerate in the 1970s, when fisheries harvesting peaked and began to linger. The marine animals under threat are mainly large animals at the top of the food chain. The populations of *C. undulatus*, a large fish in coral reefs, have rapidly declined because of heavy fishing and habitat destruction [8].

Fluctuations in population size can affect genetic diversity [9], and very small populations tend to cause inbreeding within the population [10], which can lead to a reduction in population fitness. Inbreeding decline is less pronounced in better environments but is readily apparent in harsh environments [11]. Current scientific studies have identified

a dramatic decline in the size of *C. undulatus* populations [12]; however, the role of coral reef destruction and human fishing is not clear. Assessing changes in genetic diversity and inbreeding levels is a practical monitoring tool [13]. Current molecular markers used to monitor genetic diversity include single-nucleotide polymorphisms (SNPs) [14] and microsatellites, which are also called simple sequence repeats [15]. For monitoring specific populations, microsatellite markers have the advantages of abundant alleles at individual loci [16], low typing cost [17], and mature technology [18]. They have a wide range of applications in genetic mapping, population structure analysis, genetic diversity studies, and germplasm conservation studies [19–22]. Multiplex polymerase chain reaction (multiplex PCR) refers to the simultaneous amplification of multiple target sequences by adding two or more pairs of primers to the same PCR reaction system [23]. Multiplex PCR can increase the number of microsatellite markers detected in a single run, simplifying the test procedure and reducing the cost and amount of DNA used in the sample [24,25]. In this study, 12 polymorphic microsatellite markers were selected, and one 7-plex PCR amplification system and one 5-plex PCR amplification system were successfully constructed. Then, the two systems were used to examine the genetic diversity of 30 *C. undulatus* specimens. This is the first multiplex PCR amplification system for the *C. undulatus*, providing technical support for germplasm identification, genetic diversity analysis and assessment of the effects of accretion and release in this species.

2. Materials and Methods

2.1. Sample Collection

In the present study, 30 *C. undulatus* specimens from the South China Sea were studied. The fins were first cut and immediately stored in 95% ethanol, then replaced twice with 95% ethanol, and finally stored at −20 °C for backup.

2.2. Experimental Methods

DNA Extraction and Sequencing

Genomic DNA was extracted using a Marine Tissue Genomic DNA Extraction Kit (Mobio, Guangzhou, China), and its concentration was measured using a UV spectrophotometer after DNA extraction. The concentration was adjusted to 50 ng/μL and the DNA was stored at 4 °C. Samples from 12 randomly selected individuals were sequenced on the Illumina NovaSeq platform at the Institute of Bioinformatics (Beijing, China).

2.3. Primer Design

Multiplex PCR primers were designed using the MultiplexSSR pipeline, as described by Guo and Yang [26]. The resequencing data from 12 randomly selected individuals were processed using this pipeline. The amplicons had a minimum length of 80 bp, maximum length of 480 bp, and minimum spacing of 20 bp. The minimum length of repeat units, minimum number of genotyped individuals, minimum number of alleles, and minimum depth of genotypes were set to 3, 5, 5, and 1, respectively. The optimum annealing temperature was set at 60 °C.

2.4. Primer Selection

After Tandem repeat detection, a total of 13,264 SSRs were obtained. Then, after lobSTR processing, a total of 145 high quality SSRs and ranges were finally selected. Based on the predicted results of the SSRs, 12 pairs of specific primers were selected from the developed primer sequences, namely primer pairs Cun463, Cun378, Cun500, Cun626, Cun672, Cun586, Cun148, Cun752, Cun230, Cun27, Cun485, and Cun484 following the principle of high polymorphism at the loci. Each primer pair consisted of one forward primer and one reverse primer. The dosage ratio of the forward and reverse primers was 1:4 [27]. Furthermore, two fluorescently labeled universal primers M13 and PQE-F were included: the fluorescent marker that is compatible with the universal primer M13 is 5-FAM, and the fluorescent marker that is compatible with the universal primer PQE-F

is 5-HEX [28]. The 12 primer pairs were divided into two groups: G1 group primers, including Cun463, Cun378, Cun500, Cun626, Cun672, Cun586, and Cun148, and G2 group primers, including primer pairs Cun752, Cun230, Cun27, Cun485, and Cun484. Primers were synthesized by Beijing Rui Bo Xing Ke Biotechnology Co. Site names and primer sequences are listed, and the generic primer names and primer sequence information are shown in Table 1.

Table 1. Information about site combination and primer concentration of Multiplex PCR1 and Multiplex PCR2.

Name	Number	Primer Pairs	Repetitive Units	Fragment Length Range (bp)	Primer Sequences	Primer Number
Multiplex PCR1	1	Cun463	CTAT	129–141	tgtaaaacgacggccagtcatgaaacaacccgtataccct aatagccctgctccatacttca	Cun463.F Cun463.R
	2	Cun378	AGAT	155–159	ttgagaggatcgcatccatgtattgatcatgctttctgcc taagtctgagccaatcgcatta	Cun378.F Cun378.R
	3	Cun500	GATA	297–313	tgtaaaacgacggccagtaacacaacacgcagcttagaga aatggatcctttgagagcgata	Cun500.F Cun500.R
	4	Cun626	ATA	403–409	tgtaaaacgacggccagtctatttcctgcatgtctctccc atggcccgtttatagacacaat	Cun626.F Cun626.R
	5	Cun672	TAGA	129–141	ttgagaggatcgcatccacacttcatctgtcccaccatta ctccctcctgcttcactgtact	Cun672.F Cun672.R
	6	Cun586	AGAT	301–321	tgtaaaacgacggccagtcaagaattgacaaggtttccct taactgcagtgatgaaccctgt	Cun586.F Cun586.R
	7	Cun148	ATCT	462–490	tgtaaaacgacggccagttgcaagagcattggtgatattc aagggacaacaaggacactgtta	Cun148.F Cun148.R
	8 9	Generic primers			FAM-5′-tgtaaaacgacggccagt HEX-5′-ttgagaggatcgcatcca	M13 PQE-F
Multiplex PCR2	1	Cun752	ACAAA	157–167	tgtaaaacgacggccagttctggaagcacctcatgataga ctttgactgcaaggtttcctct	Cun752.F Cun752.R
	2	Cun230	ATAG	252–272	tgtaaaacgacggccagtattaaacgcgctggttgttatt accaccaactgtgtgaatgttt	Cun230.F Cun230.R
	3	Cun27	GATA	367–375	tgtaaaacgacggccagtctctgtgctctcttgtcattgg gtcatgtcattgcatctcacct	Cun27.F Cun27.R
	4	Cun485	TGGA	404–412	ttgagaggatcgcatccacaggctaggaaggaagaaatca tttatgcctctgtgacagcatt	Cun485.F Cun485.R
	5	Cun484	GATA	470–486	ttgagaggatcgcatccacatgtatactctgccaccctcca agaagttgccaggaaattggta	Cun484.F Cun484.R
	6 7	Generic primers			FAM-5′-tgtaaaacgacggccagt HEX-5′-ttgagaggatcgcatcca	M13 PQE-F

2.5. Establishment of a Microsatellite Multiplex PCR Amplification System

The reaction volume for multiplex PCR in both G1 and G2 groups was 25 µL. Specific information is shown in Table 2. For PCR amplification, the amplification procedure used was as follows: 98 °C for 10 s, 57 °C for 40 s, 72 °C for 60 s, 35 cycles; 98 °C for 10 s, 53 °C for 40 s, 72 °C for 60 s, 15 cycles; and 72 °C for 30 min for extension [26]. After PCR, 5 µL was taken on an agarose gel to detect bright bands of the desired size. Gel electrophoresis was used to confirm that the primers amplified the target bands. The remaining samples were sent to a commercial company (Beijing Ruibio BioTech Co., Ltd., Beijing, China) for genotyping using an ABI 3730XL sequencer.

Table 2. Group G1 and Group G2 multiplex PCR reaction system.

G1 Group PCR System Reactants	Content (µL)	G2 Group PCR System Reactants	Content (µL)
Chun463.F (20 µM)	0.06	Cun752.F (10 µM)	0.06
Chun463.R (20 µM)	0.24	Cun752.R (10 µM)	0.24
Chun378.F (20 µM)	0.06	Cun230.F (20 µM)	0.06
Chun378.R (20 µM)	0.24	Cun230.R (20 µM)	0.24
Chun500.F (10 µM)	0.06	Cun27.F (10 µM)	0.06
Chun500.R (10 µM)	0.24	Cun27.R (10 µM)	0.24
Chun626.F (20 µM)	0.06	Cun485.F (10 µM)	0.06
Chun626.R (20 µM)	0.24	Cun485.R (10 µM)	0.24
Chun672.F (10 µM)	0.06	Cun484.F (10 µM)	0.06
Chun672.R (10 µM)	0.24	Cun484.R (10 µM)	0.24
Chun586.F (10 µM)	0.06	M13 (10 µM)	0.36
Chun586.R (10 µM)	0.24	PQE-F (10 µM)	0.36

Table 2. *Cont.*

G1 Group PCR System Reactants	Content (μL)	G2 Group PCR System Reactants	Content (μL)
Chun148.F (20 μM)	0.06	BSA (2 mg/mL)	0.45
Chun148.R (20 μM)	0.24	DNA (50 ng/μL)	2.0
M13 (10 μM)	0.36	Taq HS (Takara)	12.5
PQE-F (10 μM)	0.36	ddH2O	7.83
BSA (2 mg/mL)	0.45	Total	25.0
DNA (50 ng/μL)	2.0		
Taq HS (Takara)	12.5		
ddH2O	7.23		
Total	25.0		

2.6. Polymorphism Analysis

The amplification products were subjected to capillary electrophoresis usingABI3730XL. Peak types were converted to alleles using GeneMarker (version 2.2.2.0, SoftGenetics, Pennsylvania, USA) [29]. Genotype data were counted using GenAlex (version 6.5, ANU, Canberra, Australia) [30] and population genetics parameters were calculated including the number of individuals (N), number of different alleles (Na), number of effective alleles (Ne), observed heterozygosity (Ho), expected heterozygosity (He), unbiased expected heterozygosity(uHe), fixation index (F) and Hardy–Weinberg equilibrium (HW). The null allele frequency (F(null)) and polymorphism information content (PIC) were calculated using the software Cervus (version 3.0.7, Field Genetics, London, UK).

3. Results

3.1. Multiplex PCR System Establishment

The optimal amplification temperature for 12 microsatellite loci was first determined using gradient PCR amplification, and the results showed that the optimal annealing temperature was 53 °C. Two multiplex PCR systems, multiplex PCR1 and multiplex PCR2, were constructed using 12 pairs of microsatellites based on the annealing temperature and amplification length. Information on the combination of sites, primer concentrations used for multiplex PCR and information on the two universal primers and the fluorescent markers compatible with the universal primers is shown in Table 1. The results of PCR amplification and agarose gel electrophoresis of samples from 30 individuals showed that the PCR target bands were clear and did not overlap. The amplification efficiency of each locus was similar, and the amplified fragment sizes were as expected. The capillary electrophoresis diagram is shown in Figures 1 and 2.

3.2. Polymorphism Analysis

Thirty *C. undulatus* specimens were selected for PCR amplification, capillary electrophoresis, and genotyping of 12 loci in the two multiplex PCR systems, using fluorescently labeled primers. The genetic parameters were calculated and are presented in Table 3. The number of alleles of the 12 microsatellite markers ranged from 2 to 8. Among these, locus Cun148 had the highest number of alleles (8). The mean He was 0.594, and the mean Ho was 0.475. The null allele frequencies ranged from −1.104 to 0.716, with 2 loci having higher frequency invalid alleles (F(null) > 0.2), at Cun752 and Cun27. The polymorphic information content (PIC) ranged from 0.422 to 0.746, with a mean value of 0.557. A total of 9 loci were highly polymorphic (PIC $\geq$ 0.5) and 3 loci were moderately polymorphic (0.25 $\leq$ PIC < 0.5). Five loci deviated from Hardy–Weinberg equilibrium (HWE, $p < 0.05$ after Bonferroni correction) The above results show that the multiplex PCR method consisting of 12 primer pairs of microsatellites is stable and accurate in the population typing of *C. undulatus*. This can provide accurate and reliable genetic information for *C. undulatus* germplasm identification, family lineage management, and stocking effect evaluation.

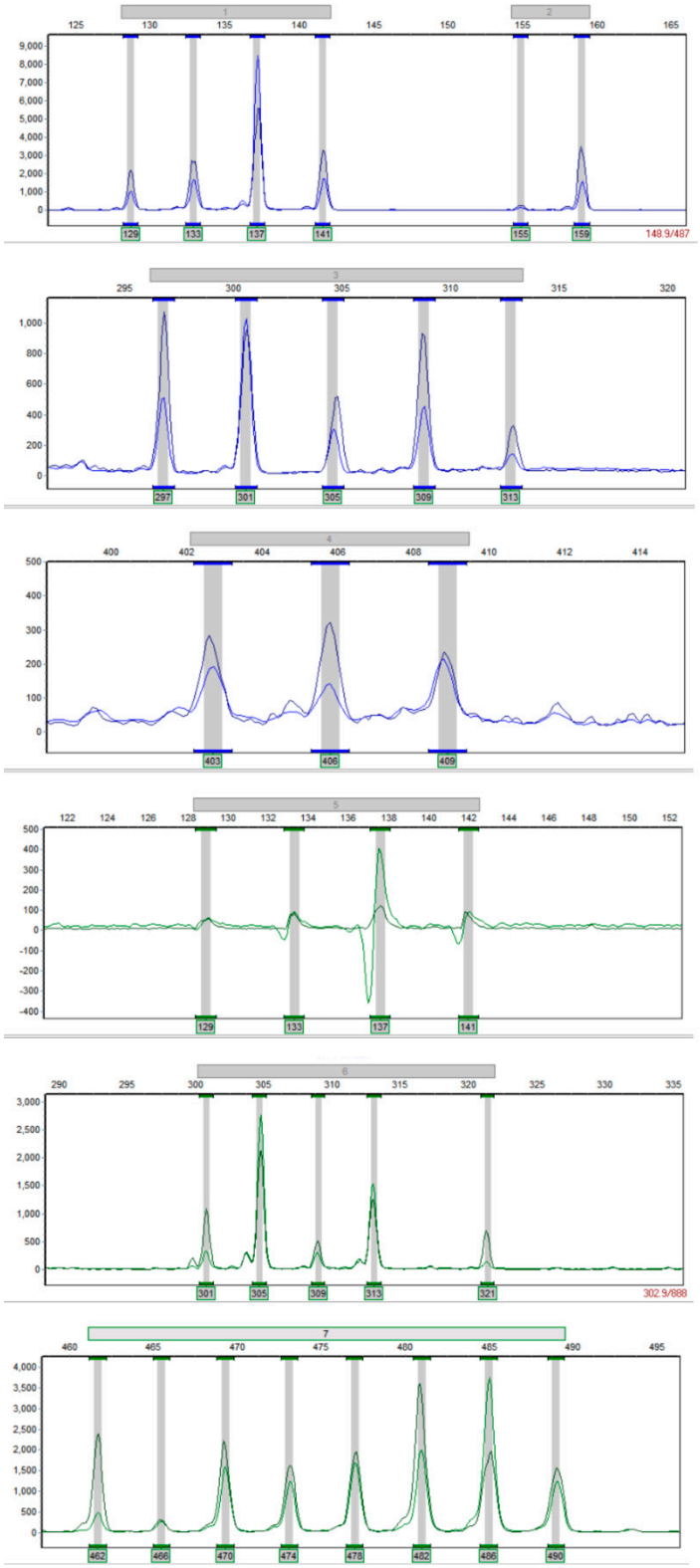

Figure 1. The capillary electrophoresis diagram of the seven primer pairs in group G1. (The 1 to 7 in this Figure correspond to alleles loci Cun463, Cun378, Cun500, Cun626, Cun672, Cun586 and Cun148, respectively).

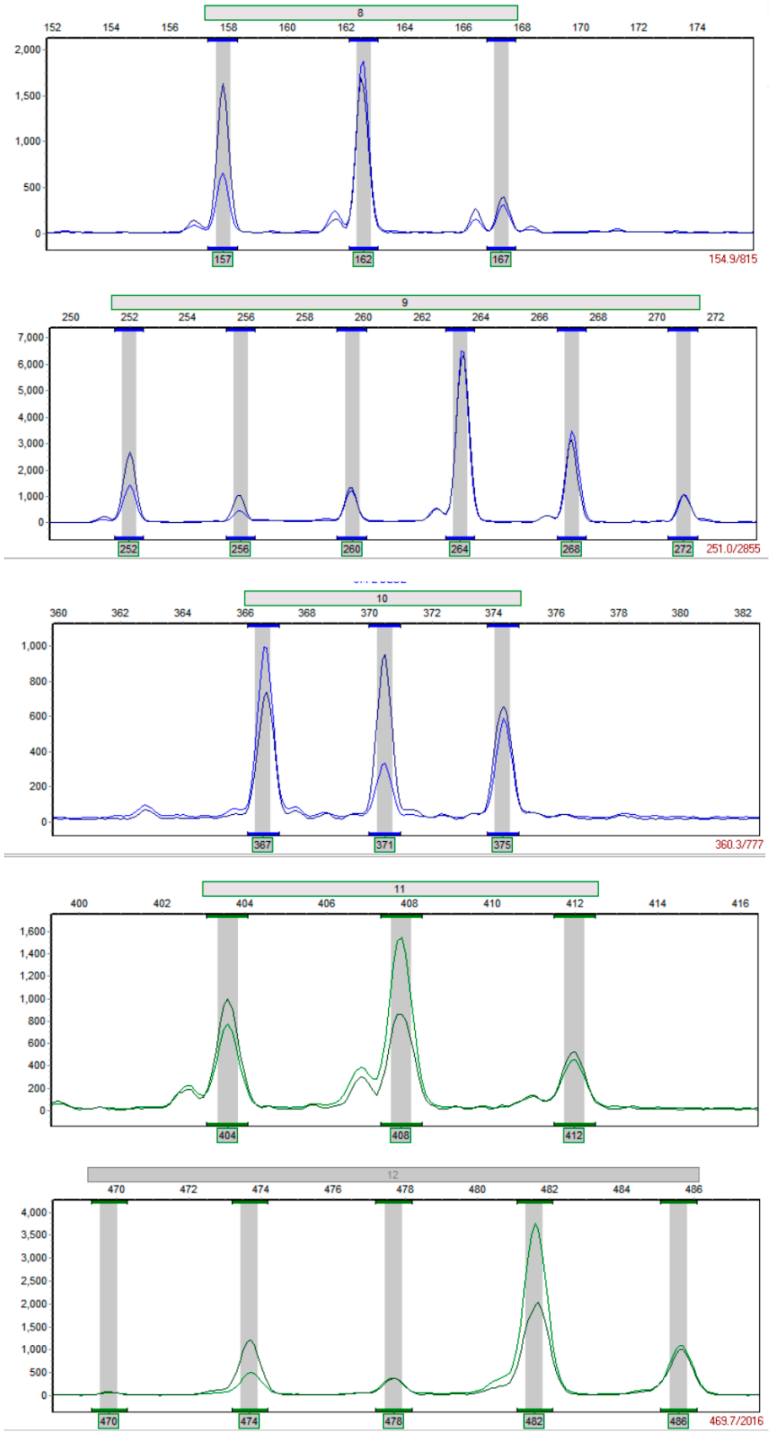

Figure 2. The capillary electrophoresis diagram of the five primer pairs in group G2. (The 8 to 12 in this Figure correspond to alleles loci Cun752, Cun230, Cun27, Cun485 and Cun484, respectively).

Table 3. Genetic parameters of 12 microsatellite loci in *C.undulatus*.

Locus	N	Na	Ne	Ho	He	uHe	F	F (Null)	PIC	HW
Cun463	30	4.000	1.835	0.367	0.455	0.463	0.194	0.125	0.422	ns
Cun378	30	2.000	1.724	0.267	0.420	0.427	0.365	0.189	0.460	ns
Cun500	30	5.000	3.455	0.667	0.711	0.723	0.062	0.039	0.671	**
Cun626	30	3.000	2.817	0.433	0.645	0.656	0.328	0.192	0.572	ns

Table 3. *Cont.*

Locus	N	Na	Ne	Ho	He	uHe	F	F (Null)	PIC	HW
Cun672	30	4.000	1.850	0.433	0.459	0.467	0.057	0.039	0.431	ns
Cun586	30	5.000	2.521	0.500	0.603	0.614	0.171	0.103	0.542	ns
Cun148	30	8.000	4.380	0.900	0.772	0.785	−0.166	−0.104	0.746	**
Cun752	30	3.000	2.203	0.300	0.546	0.555	0.451	0.256	0.486	**
Cun230	30	6.000	4.592	0.667	0.782	0.795	0.148	0.112	0.745	ns
Cun27	30	3.000	2.436	0.100	0.589	0.599	0.830	0.716	0.542	**
Cun485	30	3.000	2.332	0.500	0.571	0.581	0.125	0.071	0.536	ns
Cun484	30	5.000	2.320	0.567	0.569	0.579	0.004	−0.102	0.534	*
Mean	30	4.250	2.705	0.475	0.594	0.604	0.214	0.136	0.557	–

Note: ns = not significant ($p > 0.05$), *. Significant difference ($p < 0.05$), **. Extremely significant difference ($p < 0.01$).

4. Discussion

C. undulatus is one of the most valuable and expensive fish species in coral reefs [5], and its populations have experienced a dramatic decline [12]; however, the role of coral reef destruction and human fishing remains unclear. Assessing changes in genetic diversity and inbreeding levels is a very valuable monitoring tool [13]. For monitoring specific populations, microsatellites have several advantages and a wide range of applications. Multiplex PCR is a cost-effective and rapid method for obtaining accurate genetic information [31].

Here, we selected 12 polymorphic microsatellites, developed one 7-plex PCR amplification system and one 5-plex PCR amplification system, and used them to examine the genetic diversity of 30 *C. undulatus* specimens. Three main factors, primer combination, primer concentration, and annealing temperature, were optimized, establishing an accurate, rapid, and efficient microsatellite analysis technique for this species. We obtained a total of 12 alleles; 9 of the 12 loci were highly polymorphic, 3 were moderately polymorphic, and overall had a high level of polymorphism. After Hardy–Weinberg equilibrium validation, seven loci showed no significance and five loci deviated from Hardy–Weinberg equilibrium, which may be related to the limited sample size and the presence of invalid alleles. We further calculated the null allele frequencies and two loci had high null alleles. Therefore, it was further speculated that the five loci deviated from Hardy–Weinberg equilibrium mainly due to the limited sample size. The results showed that the multiplex amplification system was stable and reliable, and the loci were highly polymorphic. This is the first multiplex PCR amplification system for the *C. undulatus*.

Microsatellite multiplex PCR technology has been used for parentage analysis of Cirrhinus molitorella [32], and is also used commercially in large-scale selection for breeding [33]. It has also been developed and utilized as a powerful and low-cost parental assignment tool to support company-breeding programs [34]. Furthermore, it has been used to identify paternity assignments in grass carp [35] and in genetic diversity studies of Portunus trituberculatus [36]. Multiplex PCR has also been applied to detect hemolytic strains in fish and fishery products [37] and to assess Lateolabrax japonicus population genetics [38]. These studies illustrate the applicability of multiplex PCR, demonstrating its advantages in terms of performance, accuracy, experimental time, and experimental cost.

The small number of samples is a limitation in this study. More samples could improve the accuracy and reliability of the data. We can be able to take more samples and redesign primers to validate loci that have high frequency of invalid alleles and deviate from Hardy–Weinberg equilibrium to ensure the accuracy and reliability of this multiplex PCR system in the future, and more parental and offspring samples can be collected for parentage identification using the 12 microsatellite loci to elucidate genealogical information for the genetic improvement of *C. undulatus*, and to provide a basis for selective breeding.

5. Conclusions

In summary, two multiplex PCR amplification systems were constructed using 12 microsatellite markers to establish a quasi-rapid and efficient microsatellite analysis technique for *C. undulatus*. The constructed multiplex amplification systems were stable, and the loci were highly polymorphic, providing a basis for germplasm identification, genetic diversity analysis, and stocking effect evaluation of *C. undulatus*. This method can be used to select a set of primers with high polymorphism and stable PCR amplification from the developed microsatellite primers and establish an accurate, rapid, and efficient microsatellite analysis technique using multiplex PCR combination, which can provide technical support for population genetics and family lineage analysis of species.

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