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Development and characterization of microsatellite loci in a threatened marine fish, *Cheilinus undulatus* (humphead wrasse)

J. Hu[1,2,3], X.P. Zhu[1,3], J. Luo[3], S.W. Yin[1], Y.H. Peng[3], Y.L. Hu[1] and F. Zhu[1,3]

[1]College of Life Sciences, Nanjing Normal University, Nanjing, Jiangsu, China
[2]Tropical Fishes Research and Development Center, South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Sanya, Hainan, China
[3]Key Laboratory of Tropical Biological Resources of Ministry of Education, Ocean College, Hainan University, Haikou, China

Corresponding author: S.W. Yin
E-mail: yinshaowu@163.com

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ABSTRACT. *Cheilinus undulatus* (humphead wrasse) is a marine fish distributed widely throughout the tropical Indo-Pacific. It has been listed as vulnerable in the IUCN Red Data Book and in CITES Appendix II four times. Fifteen microsatellite loci were isolated and characterized for this species. The number of alleles ranged from 3 to 15 per locus, and the observed and expected heterozygosity ranged between 0.0323-0.7742 and 0.2597-0.8773, respectively. The polymorphism information content ranged from 0.2353-0.8520. Four microsatellite loci deviated significantly from Hardy-Weinberg expectations. No significant linkage disequilibrium was found among any of the loci. These microsatellite loci will be useful for future investigations of genetic variation in the wrasse population.

Key words: Humphead wrasse; *Cheilinus undulatus*; Microsatellite; DNA

INTRODUCTION

The humphead wrasse (*Cheilinus undulatus* Rüppell, 1835) is a large coral reef fish distributed widely throughout the tropical Indo-Pacific. It is the largest living member of the family Labridae, with a maximum size exceeding 2 m and 190 kg (Sadovy et al., 2003). The humphead wrasse is one of the most valuable fish in the live reef food fish trade in Asia (Scales et al., 2007). This species was listed as vulnerable in the IUCN Red Data Book and in CITES Appendix II. It was classified in this way due to concerns over rapidly declining population numbers in many areas, particularly within the last decade (Sadovy et al., 2003). Until now, studies of *C. undulatus* have mainly focused on their geographical distribution, taxonomy, morphology, ecological habits, reproduction, and protection (Wada et al., 1993; Donaldson and Sadovy, 2001; Chateau and Wantiez, 2007; Pantelis et al., 2011). Little is known about genetic relationships at different geographical scales, even though this information may provide new insight for the management and construction of protected areas. The use of polymorphic DNA markers is important for developing conservation strategies. Because of the lack of DNA markers in this species, we here report 15 polymorphic microsatellite loci isolated from the genomic DNA of the humphead wrasse to facilitate population genetic studies in this species.

MATERIAL AND METHODS

Genomic DNA was extracted from fish tail fins using the standard phenol-chloroform method. This DNA was then digested with *Bsp*143I. The digested products, 400-1000 bp in size, were separated on 1.0% low melting point agarose gel. A synthesized adaptor (LinkerA: 5'-GCGGTACCCGGGAAGCTTGG-3'; LinkerB: 5'-GATCCCAAGCTTCCCGGGTACGC-3') was ligated to the size-selected DNA fragments using T4 DNA ligase. Fragments containing microsatellite DNA were enriched by hybridization with a biotinylated $(CA)_{15}$ probe followed by isolation using streptavidin-coated magnetic beads (Promega).

The microsatellite-enriched DNA was amplified with the LinkerA primer, and then the product was ligated into a pMD18-T vector (Takara). Vectors were then transformed into competent *E. coli* DH5α cells and plated onto Luria-Bertani agar plates containing 60 mg/L ampicillin, 24 mg/L isopropyl-thio-β-galactoside, and 40 mg/L X-Gal. The colonies were each subjected to 3 independent polymerase chain reaction (PCR) screenings. The first PCR was carried out using 2 universal sequencing primers on the vector (M13: 5'-CGCCAGGGTTTTCCCAGTCACGAC-3'; RV-M: 5'-GAGCGGATAACAATTTCACACAGG-3'), the second using $(CA)_{15}$ oligo-nucleotide and the primer M13, and the third using $(CA)_{15}$ oligo-nucleotide and the primer RV-M.

A colony was considered desirable for sequencing only if the PCR product amplified by both sequencing primers was different from the product amplified by either the $(CA)_{15}$ oligo-nucleotide and the forward sequencing primer or the $(CA)_{15}$ oligo-nucleotide and the reverse sequencing primer. A total of 132 colonies were selected and sequenced using an ABI 3730 analyzer (Applied Biosystems). From the 78 microsatellite sequences obtained, we ultimately identified 20 microsatellite markers using the PRIMER PREMIER 5.0 software (Rychlik and Rhoads, 1989). Five of the 20 loci either failed to amplify product or amplified non-specific products in some individuals.

To characterize the microsatellites, genomic DNA was isolated from 31 wild individuals from LingShui City, Hainan Province, 18.2346N 109.5158E. PCR amplifications were

performed under the following conditions: an initial predenaturation at 95°C for 5 min followed by 30 cycles of denaturation at 94°C for 30 s, 30 s of annealing at temperatures listed in Table 1, and extension at 72°C for 30 s, followed by a final extension at 72°C for 5 min. After PCR amplification, the obtained DNA samples were subject to electrophoresis on 8% non-denaturing polyacrylamide gels at 12 W for 3-4 h.

Genetic diversity was assessed by calculating the number of alleles per locus, observed, and expected heterozygosity, using the Popgene32 software package (Yeh et al., 1999). Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium analysis were tested using Arlequin 3.11 (Excoffier et al., 2005). Finally, PIC-CALC was used to calculate the polymorphism information content.

Table 1. Primer sequences and characterization of 15 polymorphic microsatellite loci for *Cheilinus undulatus*.

Locus	Primer sequence (5'-3')	Ta (°C)	Repeat motif	N_A	N_E	H_O	H_E	PIC	P value (HWE)	GenBank accession
A3	F: GTTCTCAGCAGCCATCCT R: CGATTAGACCCAAACCCT	56	$(AC)_7C_2(AC)_5$	3	1.3431	0.2258	0.2597	0.2353	0.66777	HQ995574
A18	F: TGCTCCAGGTTCTCATCC R: AAGCCTTCTTGCTGTTGT	58	$(CA)_{12}$	10	2.9569	0.3548	0.6727	0.6385	0.12084	HQ995581
B22*	F: AACAGTGAGTGGGGAAAA R: AAAGTGGTGCTAGTGGAA	56	$(AC)_{11}CCACC(CA)_6$	6	4.3682	0.6452	0.7837	0.7339	0.03959*	HQ995639
A63*	F: TTAGGCTGTGAAGCATTT R: GCAGATTGTTTCCTCATT	57	$(TG)_{13}…(CT)_6…$ $(CT)_9(GTCT)_3(TC)_{19}$	7	4.7811	0.7742	0.8038	0.7613	0.00512*	HQ995601
A66*	F: CATTGTTCGGAAAAGACC R: CCAGTTGGACGCCAGTAT	58	$(GT)_{13}$	6	2.5390	0.0323	0.6161	0.5726	0.01332*	HQ995603
A67	F: TTCCTGACGGCTACCTGG R: GAGTCTGCGAAGCAATAAAG	58	$(GT)_5CA(GT)_5$	6	4.1245	0.6774	0.7700	0.7025	0.15373	HQ995604
A75	F: AAGCCTCGCCAAACTAAA R: CTACTAACGTGCGCTAAAT	57	$(TG)_5TAG\,(GT)_3G_2$ $(GT)_4G_2(GT)_7$	7	3.5073	0.5484	0.7266	0.6787	0.13770	HQ995608
A101	F: CCGGTTCTGACTCTTCTC R: GGTCATTTACAACCCAAGT	58	$(TG)_9$	5	2.9615	0.7097	0.6732	0.6211	0.11440	HQ995616
A106	F: TCTGTCCCGTGTCCATCT R: CAGTTTTGACATTGTCCCA	58	$(AC)_{10}$	15	7.3080	0.6774	0.8773	0.8520	0.87594	HQ995620
A112	F: TGAGTCAGGATTAGGTGGAT R: AAGGCAGAACCCTGGAGC	58	$(GT)_8$	13	4.0463	0.3871	0.7652	0.7341	0.62642	HQ995621
A119*	F: AGCAGGGCTCAACATAACA R: TGGATTCCTGGAGTTTGC	57	$(TG)_9…(GTCT)_5$	6	4.6764	0.5806	0.7990	0.7535	0.01402*	HQ995622
A120	F: CGGGAGGGAAGAATCACA R: AACAGGCCGACACCAAGT	60	$(AC)_5$	13	4.6764	0.4194	0.7990	0.7717	0.27378	HQ995623
A151	F: CAAGGCCGACCTGTCAAA R: GGTTATCGTGGCGTGTCC	57	$(GT)_6…(TC)_5$	8	2.7855	0.0645	0.6515	0.7383	0.07287	HQ995632
B30(2)	F: AGAGTTTGGCTGTAAGGG R: AGGAATGTTGAGAATCGTG	58	$(GT)_{12}$	9	3.0900	0.3871	0.6875	0.6524	0.09602	HQ995642
T462	F: ACTTCAGTCCAACCATCTCA R: AGCACGAGCGTCAGGAAC	56	$(ATCT)_{16}$	12	6.8889	0.6452	0.8689	0.8395	0.81543	HQ995654

*Indicate a significant deviation from Hardy-Weinberg equilibrium (HWE). F = forward primers; R = reverse primers; Ta = PCR annealing temperature; N_A = number of alleles; N_E = number of effective alleles; H_O = observed heterozygosity; H_E = expected heterozygosity; PIC = polymorphism information content.

RESULTS AND DISCUSSION

The primer sequences and other characteristics are listed in Table 1. The number of alleles ranged from 3 to 15 and observed heterozygosity ranged from 0.0323 to 0.7742. Four microsatellite loci deviated significantly from Hardy-Weinberg expectations (HWE), but no deviations from HWE were detected after Bonferroni's correction ($P > 0.05$, for all 15 alleles),

indicating a significant deficit of heterozygotes. All four loci were prone to null alleles ($P < 0.05$). The presence of excess null alleles may have biased the results of the HWE test (Jones et al., 1998), or it may be related to a particularity of *C. undulatus*, which is highly restricted to the coral reef habitat and is sensitive to environmental change. No significant disequilibrium within samples was found, indicating that genotypes at pairs of microsatellites appear to be randomly associated and are inherited in a Mendelian fashion. These genetic markers will be useful for further evaluations of spatial and temporal genetic variation and conservation management of the humphead wrasse.

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