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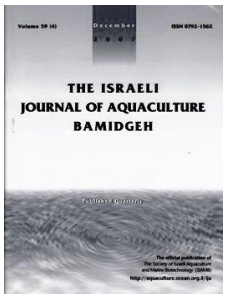

Isolation and Characterization of Polymorphic Microsatellites from Draft Genome Data for Large Yellow Croaker, *Pseudosciaena Crocea*

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Keywords: microsatellites; polymorphism; *Pseudosciaena crocea*; motif

Abstract

In this study, we isolated thirty-eight microsatellite loci from the draft genome of large yellow croaker, *Pseudosciaena crocea*. These loci were tested on 34 individuals from Zhangzhou prefecture city in Fujian. Most loci were highly polymorphic. A total of 214 alleles were detected with an average of 5.63 alleles per locus. The observed and expected heterozygosity ranged from 0.454 to 0.909 and from 0.503 to 0.918, with an average of 0.635 and 0.744, respectively. The PIC values of these loci ranged from 0.372 to 0.878, with an average of 0.712. Eight loci significantly deviated from Hardy-Weinberg equilibrium (HWE). Significant linkage disequilibrium (LD) was not found in these 38 loci. The newly identified polymorphic markers will contribute to the study of genetic diversity, population structure, and conservation of *P. crocea*.

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Introduction

Large yellow croaker (*Pseudosciaena crocea*), was once one of the four important marine fish species in China and distributed mainly in the coastal region of East Asia (Fishbase, Sang et al., 2007). Unfortunately, the population of wild large yellow croaker has severely declined due to overfishing since the 1970s (Sang et al., 2007). In order to enhance production, strict fishery management has been implemented along the Chinese coastline since the 1980s and artificial breeding methods have been developed (Hong and Zhang, 2003). However, with the development of artificial breeding of large yellow croaker, growth rate, sexual maturity, and disease resistance, have declined (Li et al., 2008). Thus, in order to improve the genetic characteristics of cultured croakers, it is necessary to conduct selective breeding (An et al., 2005). However, selective breeding techniques require a wide knowledge of the genetic diversity of this specie (Ye et al., 2012).

Microsatellites markers are suitable for population genetics and selective breeding because of their co-dominant inheritance, high mutation rates, and good reproducibility (Guo et al., 2005). Microsatellites are direct tandem repeats of short sequences of DNA (1-6bp) that commonly occur in the eukaryotic genome (Guo et al., 2013). They are widely used in population genetics, conservation, evolution, paternity determination, and stock management (Chistiakov et al., 2006; Grillo et al., 2006). In the past, the major drawbacks of microsatellite markers have been high cost, and development of species-specific markers is time consuming. In recent years, these drawbacks have been overcome with the advancement of next generation technologies (Gardner et al., 2011; Zalapa et al., 2012). Although several polymorphic microsatellite markers in the large yellow croaker have been isolated by some researchers (An et al., 2005; Guo et al., 2005; Ye et al., 2012; Lü et al., 2013), there is the need for microsatellite loci that can be used in analysis of genetic diversity and genomic mapping studies that are essential. Here, we isolated and characterized some polymorphic microsatellites from the draft genome of large yellow croaker (*Pseudosciaena crocea*). We also identified the polymorphic simple sequence repeat (SSR) markers in a population from Zhangzhou (Fujian province, China). The aim of this study was to identify polymorphic markers that contribute to the study of genetic diversity, population structure, and conservation of *P. crocea*.

Materials and Methods

Sample collection and DNA extraction

The muscle samples for genotyping procedures from individuals were collected randomly from the sea area of Zhangzhou. Muscle tissues were obtained from each individual fish, preserved in 95% ethanol and stored at -20^{0}C until DNA extraction. DNA was extracted using the standard method of standard phenol–chloroform.

The concentration of DNA was estimated by spectrophotometer (Nanodrop ND-2000, Thermo Electrom Corporation, USA) and then assessed the quality in 0.8% agarose gel.

Genomic sequence and microsatellite loci identification

The draft genome of the large yellow croaker was completed by Wu et al. (2014). Microsatellite Sequence was searched and identified with MSATCOMMANDER version 0.8.2.

SSR marker validation and population genetic analysis

Microsatellite DNA was amplified from 10 individuals using polymerase chain reaction (PCR). The amplifications were performed in a 2720 PCR machine (ABI, USA) and in a reaction mixture (25uL) containing 40-50 ng DNA, 0.2 mM dNTPs, 2 mM $MgCl_2$, 1U Taq

DNA polymerase (TaKaRa, Japan), and 0.2 μM of each primer, under the following conditions: 5 min at 94^0C, followed by 30 cycles of 40 s at 94^0C, 40s at aprimer-specific annealing temperature, 40 s at 72^0C a final extension of 72^0C for 10 min. PCR products were tested by 8% (wt/vol) polyacrylamide gels using 20bp marker under silver staining. Scorable and showed polymorphic primers were selected to be screened a second time with 32 individuals. PCR products were sequenced using an ABI 3130xl Sequencer (Applied Biosystems), and allele size was estimated using Peak Scanner Software v1.0 (Applied Biosystems).

The number of alleles (N_A), observed heterozygosity (H_o), and expected heterozygosity (H_e) was calculated with ARLEQUIN ver. 3.5.1.3 (Excoffier and Lischer, 2010). Departure from Hardy-Weinberg Equilibrium (HWE) for 38 polymorphic loci were characterized and tested using GENEPOP v.4.2 (Rousset, 2008). The polymorphism information content (PIC) of every locus was evaluated by using CERVUS version 3.0.3 (Marshall et al., 1998). Significance values processed multiple contrasts using Bonferroni corrections for necessary data (Rice, 1989). The null alleles and scoring errors were assessed using MICRO-CHECKER (Van Oosterhout et al., 2004).

Results

Bioinformatic mining of microsatellites

A total of 68008 microsatellites (2903736 bp) were identified (0.47% of the total *P. crocea* genome). Di-nucleotide repeats have the higher relative frequency with 48308 loci accounting for 70.49% (Table1), followed by 11.79% for tetra-nucleotides (8080), 8.93% for tri-nucleotides (6122), 5.74% for mono-nucleotides (3904), 2.12% for penta-nucleotides (1454) and 0.96% for hexa-nucleotides (660). Taken together, the top 20 most frequently occurring SSRs were AC, AG, TGTC, ATG, ATT, AGT, ATAG, ATGG, AGC, ACC, TAGG, GAAA, AAG, ATAC, AAGG, CCT, AACC, GATT, AATGT, ACG, in a decreasing order (Table.2). The 20 most frequently occurring microsatellites comprised 90.15% of all identified microsatellites in the *P. crocea*.

Table 1. The percentages of microsatellite motifs identified from *P.crocea* genome.

Microsatellite motif type	*Number of microsatellite*	*Percentage(%)*
mononucleotides	3904	5.74
dinucleotide	48308	70.49
trinucleotides	6122	8.93
tetranucleotides	8080	11.79
pentanucleotide	1454	2.12
hexnucleotides	660	0.96
Total	68528	100

There are large differences in the relative abundances of specific repeat motif. AC repeat (43564, 90.17%) was the most abundant of the total dinucleotides, while AG (4629, 9.6%) and AT (110, 0.22%) were much lower, and GC type was very rare (5%). Of the tri-nucleotides, ATG was the most frequent (1484, 24.2%), followed by ATT (1311, 21.4%), AGT (1072, 17.5%). The most frequent tetra-nucleotide was TGTC (3443, 42.6%). For the longer repeats of penta-to hexa-nucleotides, the most abundant motifs were AATGT (191, 13.1%) for penta-nucleotides, AGAGAC for hexa-nucleotides.

Table 2. The 20 most frequently occurring microsatellites in *P.crocea*.

SSR motifts	*Number of repeats*	*% of total SSRs*
AC	43564	64.06
AG	4629	6.8
A/T	3904	5.7
TGTC	3443	5.06
ATG	1484	2.18
ATT	1311	1.93
AGT	1072	1.58
ATAG	968	1.41
ATGG	667	0.98
AGC	655	0.96
ACC	620	0.91
TAGG	538	0.79
GAAA	467	0.69
AAG	405	0.6
ATAC	399	0.59
AAGG	386	0.57
CCT	336	0.49
AACC	258	0.38
GATT	205	0.3
AATGT	191	0.28

SSR variation and genetic diversity analysis

Among the total of 68008 microsatellite sequences, 158 microsatellite primer pairs were randomly selected for validation using 10 individuals. Ultimately, 86 out of 158 loci that were amplified produced clear bands. Of these, 48 loci were monomorphic, and 38 other loci were polymorphic. These polymorphic loci were further characterized with 32 wild-caught individuals *P. crocea* from the Zhangzhou sea area. The sequences of the 38 loci were deposited in the Genbank. Characteristics of these 38 loci are summarized in Table 3. We detected 214 alleles (N_A) at the 38 microsatellite loci, and the number of alleles per locus ranged from 5 to 12, with an average of 5.6. Observed heterozygosity (H_o) and excepted heterozygosity (H_e) ranged from 0.454 to 0.909 (averaged=0.635) and 0.503 to 0.918 (averaged=0.744), respectively. The PIC of each locus raring from 0.372 to 0.878 (averaged=0.712). All but six microsatellites were highly informative (PIC>0.5), according to the criterion proposed by Botstein et al. (1980). Eight loci (Loci3, 5, 25, 26, 53, D64, D75, D76) deviated significantly from HWE after a sequential Bonferroni correction ($p<0.05$). MICRO-CHECKER testing showed no existence of null alleles, large allelic dropout or scoring errors in these 38 loci (Van Oosterhout et al., 2006). After Bonferroni correction, no linkage disequilibrium was detected among the loci.

Table 3. Microsatellite loci developed for large yellow croaker(*Pseudosciaena crocea* Richardson).

Loci	*Motif*	*Forward primer*	*Reverse primer*	*Tm(℃)*
1FR	(AG)7	CATGTTGGTGGTGTGTAACAAT	GCTTGATTAGTGTGTATGGCTTG	54
3FR	(AG)7	GATGTAACCCGATCTCCTGAG	GCTGCGAATCAAACACCAC	52
5FR	(TG)5(CG)(TG)4	CTTGCAATGGAAGAGGACACT	GGGTTCAGCATTTACCGAAA	50
6FR	(TC)12	CCTCATTGACTCACTTGTGTG	GATCGATAGCAGCAGTTGGA	50
7FR	(CTG)7	GCTGCATGAGTGATGCCATA	GGAAAAGTGGGACATGATGC	51
16FR	(CA)14	ATAATGCAAAGGGAGAAAGGG	AAATACAGCGATGACAACAGGA	53
18FR	(TG)10	AATGCGCTGTGACTGACA	AAGCGCAGTGCATGATAAAC	51
21FR	(GT)6(TT)(GT)4	TCTACTGGGAACTTCATCAGC	CCACAACAAGAAGGCAAAGA	50
23FR	(CA)12	CATGTTGGTGGTGTGTAACAAT	GCTTGATTAGTGTGTATGGCTTG	51
25FR	(TG)9…(TG)6	TGCCATACAGGATCTCCCG	CATCAGGCTCACACACCTTG	51
26FR	(CA)5(T)(AC)9...(TG)13...(GA)6	TCAGCTGAGTAGGTCGACACTCT	CTCACCCCTTTTGCTGATCT	50
32FR	(ACTC)6(CA)4	ACTGACTCACTGACTCACTGACTC	GGGTGTGTGGGTTCACATAA	50
45FR	(AT)7(AT)7	GATCAGATGGATAGCATTAGCAG	TAATATGGTGCCCCTGCTTT	53
48FR	(AC)9...(CA)6...(CA)5	GACCCACACACACTCCTTCAT	CACGCAGGTCTCAGACTGTT	50
49FR	(TC)5…(CT)8…(CT)6	ATCTTCAGAAGGAGGCTCCTC	CAGTGGTAGTGGAACGCAAA	55
51FR	(AC)5	TTGGCAACGATGGACTGAC	GGCCTGTTTCTGCAGGTCTA	50
52FR	(AC)5(AA)(AC)5(AA)(AC)9	TGCAGGCTGACACAGGAT	AGCCTCTACCTCGGTTCACA	50
53FR	(GT)6	AGTTTGTGCAGTGCGTCGT	CCATCCACGTGACAGCTCTA	51
68FR	(AT)11	AGTAACAAAGGTGTCCGAACGTT	GTTCAACCGAAAAAGCTCTACACTA	51
73FR	(CT)10	ATCTCACCCCAGAGGAGGAA	CTCATCAGTTTCACTTAAACACTGGAC	50
77FR	(TG)9(CG)5	AGCTCTCTTGGCTTAACTAGGAGAT	AATACTTTGGAGGATTCTGGTGAC	53
D1FR	(TTA)5	TGTTGTCACACTGAGGTTTCAGTG	GTGTCTCCCTTCAAATCAAAGC	52
D2FR	(ACTC)7	GCTCTGTGCTGAATCCCAA	GTCTGCGCTGCTTTAAGGAT	52
D4FR	(ATGG)6	TGTCAGCTGGGATTGGCT	GGGCATCAGTAAGGACAGGT	54
D5FR	(CTT)9	TGTCCTTTCCAGCGTCAG	GCCTCTCACACCATCTTTCC	52
D7FR	(ATTT)6	CATGATTAATGCAGAGTGTCTGC	GCTGTGCGCTATGGAAAATAA	52
D10FR	(TTA)5	GTCAACTTGTCATTGCTCAGTT	CATGTTTTGCATTGTCACCA	57
D13FR	(CCT)4(TCTTCT)(CCT)5	GAGACAGGATGGTGTAGCTTTCC	CGAACGTGTTTCTGGAAGGT	53
D25FR	(AATC)4	TGGACACCATATAGTCCAG	GCACAATGTAAGAAGTTGGG	52
D40FR	(AGA)5	TGATTGATGCGTTCCGGTTG	TCCCTGTGTGCCTTCTTCTT	52
D49FR	(TTATC)5	TGTGACTGACTGTGCTTCCA	ACGTAGCCTATTACCCTGCA	54
D63FR	(TATTC)6	TGTTTTGTACTCCTGCACTGAC	ACATCATACAAACAGCACCAGG	52
D64FR	(TAGCC)7	GGAGGTTGTTCGGCTTTGAA	AAGCTCGCTGTTGTGTTTGA	52
D65FR	(ATGTG)5	TAAAACGTTCCCTGCCGACT	GTGCAGCATGGAAAGAGACA	54
D72FR	(TGTGC)5	CAACAACAACGACTCTTTCC	CCAAGAGTGCTTCAGTCAAC	54
D75FR	(TAGCC)7	CGGCTTTGAAGAAGTTTACA	AACAAAAATCCACCCATCTC	56
D76FR	(AATCT)4	TCACACATGTACTGCACACC	TGAGGAATTACCAGAGTTGC	52
D77FR	(TATTC)6	ACTCCTGCACTGACTATTGC	CATACAAACAGCACCAGGAT	52

Table 4. Characterization of ten microsatellite loci in large yellow croaker (*P. crocea* Richardson).

Locus	N_A	*Allele size*	H_O	H_e	*PIC*	*HWE* $_P$	*Accesion.No.*
1FR	5	170-200	0.5	0.768	0.792	0.236	KC936999
3FR	7	215-230	0.545	0.866	0.842	*	KC937000
5FR	6	165-180	0.454	0.68	0.806	*	KC937009
6FR	8	220-250	0.9	0.889	0.86	0.098	KC937010
7FR	7	235-270	0.636	0.84	0.876	0.194	KC937011
16FR	10	160-200	0.545	0.892	0.89	0.099	KC937007
18FR	6	220-270	0.727	0.779	0.78	0.945	KC937001
21FR	6	160-200	0.636	0.745	0.795	0.945	KC937002
23FR	5	98-110	0.454	0.667	0.752	0.168	KC937006
25FR	5	180-215	0.727	0.766	0.79	*	KC937003
26FR	7	145-180	0.636	0.835	0.815	*	KC937004
32FR	12	208-250	0.909	0.918	0.878	0.349	KC937012
45FR	7	220-260	0.909	0.853	0.817	0.673	KC937005
48FR	11	230-280	0.8	0.868	0.859	0.402	KC937013
49FR	9	220-260	0.909	0.9	0.859	0.773	KC937014
51FR	5	242-280	0.545	0.848	0.849	0.396	KC937015
52FR	7	242-270	0.454	0.745	0.806	0.323	KC937008
53FR	7	220-250	0.454	0.831	0.836	*	KC937016
68FR	10	160-220	0.727	0.875	0.872	0.088	KC937017
73FR	6	170-210	0.636	0.814	0.832	0.386	KC937018
77FR	7	240-270	0.6	0.832	0.861	0.24	KC937019
D1FR	4	143-170	0.687	0.89	0.864	0.06	KJ143695
D2FR	3	160-170	0.656	0.718	0.655	0.087	KJ143696
D4FR	3	240-250	0.625	0.534	0.416	0.284	KJ143697
D5FR	10	247-252	0.719	0.542	0.433	0.084	KJ143698
D7FR	3	214-236	0.812	0.879	0.851	0.369	KJ143699
D10FR	4	220-240	0.565	0.644	0.557	0.067	KJ143702
D13FR	3	213-225	0.656	0.651	0.565	0.417	KJ143705
D25FR	3	198-203	0.469	0.677	0.592	0.079	KJ143707
D40FR	2	178-185	0.531	0.669	0.584	0.055	KJ143710
D49FR	4	170-175	0.5	0.503	0.372	0.97	KJ143711
D63FR	3	164-173	0.5	0.724	0.655	0.092	KJ143712
D64FR	3	210-220	0.533	0.554	0.441	*	KJ143713
D65FR	3	160-168	0.517	0.668	0.583	0.298	KJ143714
D72FR	4	178-200	0.687	0.735	0.673	0.218	KJ466361
D75FR	4	210-235	0.552	0.564	0.44	*	KJ466362
D76FR	3	217-220	0.714	0.626	0.541	*	KJ466363
D77FR	2	155-160	0.69	0.508	0.375	0.05	KJ466364

N_A: number of alleles; H_o: Observed heterozygosity; H_e: Expected heterozigosity; PIC: Polymorphic information content; *HWE* $_P$: Hardy-Weinberg equilibrium *P* values; *: Significant deviation from *HWE*(P<0.05).

Table 5. The influence of repeat motif and repeat number on the polymorphic level of microsatellite markers.

Repeat motif	N_A	H_O	H_e	*PIC*
Dinucleotide	7.05[a]	0.639[a]	0.813[a]	0.827[a]
Trinucleotides	5[ab]	0.632[a]	0.706[ab]	0.647[b]
Tetranucleotides	4.8[ab]	0.694[a]	0.745[a]	0.678[b]
Pentanucleotides	3.25[b]	0.587[a]	0.61[b]	0.51[c]

Number of repeat motif	N_A	H_O	H_e	*PIC*
4-6	3.75[c]	0.593[a]	0.695[b]	0.625[b]
7-10	6[b]	0.628[a]	0.726[b]	0.703[b]
»11	7.82[a]	0.696[a]	0.829[a]	0.835[a]

N_A: number of alleles; H_o*:* Observed heterozygosity; H_e: Expected heterozigosity; PIC: Polymorphic information content; The different letters in the same column in the upper right corner of the table represent significant differences (P<0.05).

Discussion

Our results suggest that developing large numbers of polymorphic microsatellites using draft genome is a rapid and cost-effective genetic informative means in fish species. Di-nucleotide repeats were the most frequent SSR motif type, similar to the findings in *Catla catla* (Sahu et al., 2014) and *Tetraodon nigroviridis* (Crollius et al., 2000). Trinucleotides are the second most frequent motifs in *P. crocea*; however, more tetranucleotides than trinucleotides were observed in the genomes of Japanese pufferfish, *Fugu rubripes* (Edwards et al., 1998). Longer repeats of penta- to hexa-nucleotides covered only a small percentage of total SSRs (2.12%, 0.96%). Results showed there was a significant negative correlation between abundance and length of microsatellite repeat units, which can be useful for the selection of SSR loci and primer designing (Sahu et al., 2014). In *P. crocea* draft genome, the AC repeat (43564, 90.17%) was the most abundant of the total dinucleotides, and GC type was very rare (5). These results are consistent with data for most vertebrate species, including fish (Kang et al., 2012; Luo et al., 2012; Chistiakov et al., 2006). The low frequency of GC dinucleotides in genomes is attributed to the methylation of cytosine, which increases its chance of mutation to thymine by deamination (Schoreeret and Ggartlar, 1992).

Overall, 38 of 86 loci were found to be polymorphic. The percentage of polymorphic markers was 30.4% in this study, which was similar to previously reported results for large yellow croaker (31.4%) (Chang et al., 2009), but lower than that described for large yellow croaker (67.5%) designed by Lü et al. (2013). The mean number of alleles per locus, H_o, H_e and PIC were 5.6, 0.635, 0.744 and 0.712, respectively. Allele richness of these microsatellites was lower than that isolated for previous *Larimichthys crocea* microsatellites by Chang et al. (2009) and Ye et al. (2012) using an enriched library technique compared with the microsatellite markers developed in large yellow croaker by other researchers (Guo et al., 2005; Ye et al., 2012). The PIC value of these microsatellites was higher. Eight loci (Loci3, 5, 25, 26, 53, D64, D75, D76) deviated significantly from HWE after a sequential Bonferroni correction ($p<0.05$) (Rice, 1989). The deviation from HWE might be due to insufficient sample size, experimental artifacts, lack of random mating (Chapuis and Estoup, 2007; Rousset and Raymond, 1995).

In our study, differences in the polymorphism were found in different microsatellite types (Table 4). The polymorphism of dinucleotide was significantly higher than in pentanucleotide, although there were no marked differences in the observed heterozugosities of alleles between di-, tri-, tetra-, or penta-nucleotide ($p<0.05$). However, the loci displaying more repeat motifs accounted for higher polymorphism (Table5). ANOVA analysis showed the number of N_A, H_e, and PIC significantly increased with the increasing of the number of repeat motif. Positive correlation was found between the number of repeat motif and the number of alleles, but no statistical difference in the

number of alleles between di-, tri-, or tetra-nucleotide were reported (Rezende et al. (2010). Previous studies showed loci with maximum repeat numbers were highly polymorphic (Goldstein and Clark 1995). Because DNA slippage may increase the number of repeats this could lead to higher mutation rates (Petit et al., 2005). Combining the information of the microsatellite repeat motif and number of repeats with polymorphism may help develop efficient polymorphic microsatellites.

These new 38 microsatellite loci showed high genetic diversity and suitability for studies of genetic structure and population ecology. Therefore, these microsatellites provide a foundation for research into protecting genetic resources and breeding programs of this species.

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