Polymorphic microsatellite loci developed from the Hong Kong oyster (*Crassostrea hongkongensis*)

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Genet. Mol. Res. 15 (4): gmr.15048676
Received March 30, 2016
Accepted May 6, 2016
Published October 5, 2016
DOI http://dx.doi.org/10.4238/gmr.15048676

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ABSTRACT. Forty polymorphic microsatellite loci were developed from *Crassostrea hongkongensis* using an enriched partial genomic library with magnetic beads. The polymorphism of these loci was assessed in 30 individuals from a wild population. The allele number of the polymorphic markers ranged from 2 to 13, with an average of 5.8 per locus. The polymorphism information content ranged from 0.032 to 0.891 and 37 loci presented a medium or high level of polymorphism. The observed and expected heterozygosity values

ranged from 0.033 to 1.000 and 0.033 to 0.931, respectively. Of the 40 loci, 28 were found to conform to Hardy-Weinberg equilibrium (HWE), whereas the remaining 12 showed a significant departure from HWE. The availability of these markers will aid future genetic studies in *C. hongkongensis*.

Key words: *Crassostrea hongkongensis*; Microsatellite loci; Polymorphism

INTRODUCTION

The Hong Kong oyster (*Crassostrea hongkongensis*) is an economically important marine species that primarily occurs in waters along the coast of the South China Sea (Lam and Morton, 2003). In recent years, the rising consumption of oyster products and the decline of natural resources have led to increasing aquaculture practices of this species. Oyster farming of *C. hongkongensis* has been supporting one of the largest marine aquaculture industries in this area (Yu et al., 2008). As the extensive cultivation practice has proceeded, farming of *C. hongkongensis* has experienced some problems, including a decline in wild seed quantity and quality, and increased seasonal mortality (Xia et al., 2009). Therefore, enhanced stock management and genetic improvement of this species are greatly desired in the industry. Microsatellite markers are useful for the applications mentioned above (McGoldrick et al., 2000; Liu and Cordes, 2004). However, few microsatellite loci have been reported in this species (Xia et al., 2009; Li and Yu, 2010; Xiao et al., 2011; Zhao et al., 2015) and more loci are required to enable further work such as genetic mapping and trait improvement studies. Here, we described the development and characterization of 40 new polymorphic microsatellite loci for *C. hongkongensis*, which will be a useful tool in future studies of this species.

MATERIAL AND METHODS

A microsatellite-enriched genomic library was constructed following the protocol described by Carleton et al. (2002). Genomic DNA for constructing the enriched library was extracted from ethanol-preserved muscle obtained from Zhuhai Hengqin Island, Guangdong Province, using standard proteinase-K digestion and a phenol/chloroform protocol with RNase treatment. Genomic DNA was digested with restriction enzyme *Sau*3AI (Promega, Madison, WI, USA), and 400- to 1000-bp fragments were recovered from a 1% agarose gel using the MinElute Gel Extraction Kit (TaKaRa, Dalian, China). Fragments were then ligated to a blunt-end adapter (SAULA: GATCGTCGACGGTACCGAATTCT, SAULB: GTCAAGAATTCGGTACCGTCGAC) with T4 DNA ligase (TaKaRa). The ligation products were amplified by polymerase chain reaction (PCR) using the adapter SAULA as primers. Enrichment was performed using the Streptavidin MagneSphere Paramagnetic Particles Kit (Promega) with biotinylated probes $(CAT)_{12}$GCTTGA-Biotin and $(GAT)_{12}$GCTTGA-Biotin. Amplified fragments were denatured and hybridized to biotin-labeled dinucleotide repeats in 6X SSC/0.1% SDS at 58°C for 1 h. The DNA that had hybridized to the probes was then captured on streptavidin-coated magnetic beads and washed to remove unbound fragments. To release the probe, the enriched DNA was

denatured in 0.1X TE at 95°C, and was subsequently amplified with PCR using SAULA as primer. To achieve sufficient flanking region sequences, PCR products ranging from 400 to 900 bp were excised from the agarose gel and recovered. These fragments were ligated with pMD19-T (TaKaRa) at 16°C for 10 h. Positive clones were identified from the recombinant colonies via PCR using M13 primers and $(CAT)_{12}$ for CAT-repeats and $(GAT)_{12}$ for GAT-repeats. The positive clones that showed double or multiple bands were cultured overnight in LB medium containing ampicillin and sequenced with an ABI 3730 XL (Applied Biosystems, Foster City, CA, USA).

We detected 170 microsatellite-containing sequences, 98 of which contained sufficient flanking sequence for primer design. The designed primers were evaluated using 30 individuals from a wild population collected from Zhuhai Hengqin Island, Guangdong Province. The PCR was performed in a volume of 10-μL reactions containing 0.25-0.5 U *Taq* polymerase (Tiangen, Beijing, China), 1X PCR buffer, 1.0-2.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.2-1 μM each primer, and 20-100 ng total DNA. The PCR conditions were as follows: initial denaturation at 95°C for 5 min; 30 cycles of 94°C for 30 s, a primer-specific annealing temperature for 30 s, and 72°C for 1 min; followed by a final extension at 72°C for 10 min. The PCR products were examined with electrophoresis on 8% non-denaturing polyacrylamide gel and visualized with silver staining. A 10-bp DNA size standard (Invitrogen, Carlsbad, CA, USA) was used as a reference marker for allele size determination. Allele size was determined using the software Gel-Pro Analyzer v. 4.5. The polymorphism information content (PIC) was estimated with PIC-CALC v. 0.6. The number of alleles, heterozygosity, deviation from Hardy-Weinberg equilibrium, and linkage disequilibrium were analyzed using GENEPOP v. 3.4 (Raymond and Rousset, 1995).

RESULTS AND DISCUSSION

Of the 98 primers designed, 76 consistently generated clear and specific products, and 40 primers were polymorphic. Of the 40 polymorphic loci, twenty-six loci were perfect tri-nucleotide repeats or compound motifs, whereas fourteen loci were di-nucleotide repeats (Table 1). The allele number of these markers ranged from 2 to 13 with an average of 5.8 per locus. The locus *Ch*521 was the most polymorphic with 13 distinct alleles, whereas four loci were the least polymorphic with 2 alleles. The PIC ranged from 0.032 to 0.891 and 37 loci presented a medium or high level of polymorphism according to the Weber (1990) standard. The observed and expected heterozygosities ranged from 0.033 to 1.000 (mean 0.590) and 0.033 to 0.931 (mean 0.675), respectively (Table 1). Twelve loci (*Ch*501, *Ch*505, *Ch*507, *Ch*512, *Ch*514, *Ch*516, *Ch*519, *Ch*521, *Ch*526, *Ch*527, *Ch*535, and *Ch*540) showed significant deviations in the observed genotype frequencies from Hardy-Weinberg equilibrium, after Bonferroni correction. Six pairs of loci (*Ch*524 and *Ch*506, *Ch*514 and *Ch*533, *Ch*514 and *Ch*535, *Ch*535 and *Ch*533, *Ch*534 and *Ch*517, and *Ch*516 and *Ch*517) showed significant linkage disequilibrium ($P < 0.01$). In addition to the published microsatellite markers, 214 SNPs have been found to be transferable to *C. hongkongensis* (Zhong et al., 2014). All available microsatellite and SNP markers will be useful for linkage mapping and genetic improvement of this species.

Table 1. Characterization of 40 microsatellite loci in *Crassostrea hongkongensis*.

Locus/accession No.	Primer sequence (5'-3')	Ta (°C)	Repeat motif	Size range (bp)	N_A	H_O	H_E	PIC	HWE P value
*Ch*501/ KT021728	F: GCGTGTAACTGCATTCCT R: CCTGATAAGAGCGGGTTT	64	$(CA)_{26}$	241-286	5	0.231	0.695	0.6261	0.0000
*Ch*502/ KT021729	F: CACTTCATCTTCCATCAC R: ATTCTACGACAAGGTCTC	58	$(TGA)_4$	186-226	5	0.842	0.708	0.6296	0.0057
*Ch*503/ KT021730	F: TGATGATTACAGTTCTCC R: GAGTTGCGACAAATAGAT	59	$(TCA)_{10}…(CAA)_8$	151-204	6	0.923	0.817	0.7712	0.6246
*Ch*504/ KT021731	F: GCCGACCTACTAAACAAG R:GTACAACCCATCCACGAA	64	$(GA)_8$	255-329	7	0.900	0.807	0.7646	0.5839
*Ch*505/ KT021732	F:AGCAAACTTGTAATAGGC R:GATCATCTCCATCATCAT	58	$(ATC)_5$	164-242	6	0.262	0.738	0.6743	0.0000
*Ch*506/ KT021733	F:AGAAAGGAGATACCGAGA R:TTTCCACATGAGGTAGGG	56	$(CTG)_{10}…(CTG)_6…(TGC)_8$	261-357	8	0.933	0.810	0.7745	0.0058
*Ch*507/ KT021734	F:GGAGAATAGAAACCCAAG R:ACGACTCGCACAGTAATT	58	$(TTC)_4…(CTT)_5$	382-435	3	0.585	0.931	0.4816	0.0000
*Ch*508/ KT021735	F:AGAAAGGCGAAGTAATGC R:CACCATCTGTCATCATCA	54	$(CAT)_{11}$	356-419	5	0.809	0.828	0.7627	0.0630
*Ch*509/ KT021736	F:AAGCAGCAGTAACACCAG R:CCCTTTCAACTCATCCTC	50	$(TCC)_6…(TCC)_7$	286-327	4	0.483	0.409	0.3755	0.8722
*Ch*510/ KT021737	F:CATCTACTGTGGCTGTTT R:GGATGATGACCATGATGA	58	$(GAT)_5…(GAT)_4$	262-327	4	0.620	0.588	0.5216	0.4948
*Ch*511/ KT021738	F:CCGATACATTGGCAGTAA R:AGATACCGGGAATTCACA	58	$(CAG)_8…(CAG)_6…(CAG)_{10}$	251-345	12	0.966	0.853	0.8238	0.1162
*Ch*512/ KT021739	F:ATGACGAAATACCTGACC R:TTTATCATCTGGAACCCC	56	$(CAT)_4$	166-198	5	0.764	0.857	0.7040	0.0000
*Ch*513/ KT021740	F:AGTGGGCTAACTGTATCA R:TGCGAAAATGGTTGCTGC	58	$(GA)_{30}$	243-368	4	0.551	0.567	0.4528	0.0022
*Ch*514/ KT021741	F:TCTGGAACACCGTCATTA R:ATGAAGACGACGACAATG	58	$(GAT)_5$	258-306	3	0.312	0.635	0.5539	0.0000
*Ch*515/ KT021742	F:GGACAGGCAGTCTGTTAG R:CATTGGCTAGAGGTTGTA	56	$(CAT)_4$	301-322	2	0.297	0.231	0.2120	0.1677
*Ch*516/ KT021743	F:ACAACTGCATGTTTCTGA R:CTGGAAAGATTTTGTGAG	58	$(GAT)_5…(TGA)_4$	337-425	6	0.552	0.805	0.7605	0.0000
*Ch*517/ KT021744	F:AACCATTGATAGGCGACA R:ACGGCACAGAGGAAACCA	52	$(CAT)_8$	283-319	6	0.896	0.775	0.7283	0.0981
*Ch*518/ KT021745	F:ACCTCTACTTCCCCGACCAC R:AGCATGAGGGTGAGGAGAGA	60	$(CT)_7$	241-287	2	0.091	0.091	0.0831	0.9340
*Ch*519/ KT021746	F:GTACCAACCCATAGAAAG R:GAAGAGGCTGCTAAGAAA	58	$(ATT)_4$	204-249	2	0.033	0.305	0.2546	0.0000
*Ch*520/ KT021747	F:GATGATGTGATGATGTGG R:TAGCGGTACCCTTGTTAA	56	$(CAT)_{15}$	308-342	4	0.823	0.718	0.6423	0.8304
*Ch*521/ KT021748	F:GGAAAACGTGGACAATTA R:GATCGTATGATCTCAACA	54	$(GT)_{40}(GA)_{19}$	252-358	13	0.400	0.918	0.8906	0.0000
*Ch*522/ KT021749	F:CAAGGAGGGATTCAAGGA R:ACGGAAAAGTTCAGAGCG	58	$(GAA)_{12}$	209-243	2	0.033	0.033	0.0323	0.9230
*Ch*523/ KT021750	F:GACATGCCGCAGACACTTT R:GGCAAGTATAACGATGGCACT	69	$(CA)_{35}$	99-187	11	1.000	0.885	0.8506	0.2062
*Ch*524/ KT021751	F:TTGTAATGGGTGATGACG R:ACAACGCTCACAACAACA	60	$(AAC)_5$	214-269	4	0.429	0.566	0.4989	0.0105
*Ch*525/ KT021752	F:TTCCAGGTCATCTCCAAG R:CACTCCTACCCTCATAAA	58	$(CA)_{27}$	162-214	6	0.435	0.772	0.7173	0.0017
*Ch*526/ KT021753	F:TGCCCAATCACAGGACAT R:ACATGCAACTACAACCCC	66	$(CAT)_7$	184-228	10	0.633	0.869	0.8376	0.0000
*Ch*527/ KT021754	F:ACTGCGTGCAATAAAACC R:GTCTCCTTGTCATCCTCA	58	$(TCA)_5$	192-216	7	0.552	0.834	0.7946	0.0000
*Ch*528/ KT021755	F:GACCAGCTTTGAGAACAT R:CTTACTAACACTTACCCTACC	64	$(TC)_6$	230-259	3	0.200	0.371	0.3095	0.0020
*Ch*529/ KT021756	F:CCCTCTCTTTTTCTGTCTCTGTTA R:GCTTTTTATACACAGGCATCTGAG	58	$(CT)_7…(CT)_{30}$	104-173	8	0.825	0.827	0.7857	0.2968
*Ch*530/ KT021757	F:TTTCTATGCCCACCCACT R:TCAATCTTACCGCACGAC	50	$(CAT)_5…(ATC)_4$	251-279	4	0.633	0.525	0.4378	0.0241
*Ch*531/ KT021758	F:GCATCCCAATAGCACATC R:ACCTCAACCCATCTTTCC	58	$(GT)_5$	258-306	6	0.866	0.735	0.6760	0.0086
*Ch*532/ KT021759	F:TCTACAAAGGCTACTCAC R:AACCCTGATAATCCTACC	58	$(CT)_{14}$	298-347	6	0.643	0.721	0.6542	0.2701
*Ch*533/ KT021760	F:TACAACCTCTAGCCAATG R:TCAACCAATATGCCACCA	50	$(CT)_5$	251-286	4	0.766	0.575	0.4993	0.0432
*Ch*534/ KT021761	F:GACAGGGGATACTAAAGC R:GACCCTTAGATTCACTCC	58	$(CAT)_4…(CAT)_8$	318-367	9	0.798	0.896	0.7560	0.0057
*Ch*535/ KT021762	F:CAATCTTACCGCACGACA R:ATTTCTATGCCCACCCACTT	50	$(GAT)_4…(GAT)_5$	252-278	5	0.366	0.703	0.6419	0.0000
*Ch*536/ KT021763	F:TTACAAATGTTCTGTGGC R:GGATAATGTTGCTGGTCT	50	$(ATC)_8$	313-356	5	0.643	0.719	0.6492	0.0728
*Ch*537/ KT021764	F:AGATGGGTCTGCTTCACA R:GCCCTCTGGCTTGATTTA	58	$(CA)_5CG(CA)_{18}$	252-346	9	0.875	0.897	0.8552	0.0141
*Ch*538/ KT021765	F:CTTAGGTGGAAGCAAGTC R:AACCCACATCGCAAATAC	50	$(GA)_8…(GA)_7$	255-328	8	0.654	0.692	0.6115	0.7010
*Ch*539/ KT021766	F:GTCTGAATCTTCCTGGTT R:GGCATTCCTGATTACAAG	58	$(ATG)_4…(ATG)_4$	253-289	8	0.666	0.545	0.5154	1.0000
*Ch*540/ KT021767	F:CCCATGACAGGGGATACT R:TGTCCTCTGTTGGGTCTG	60	$(CAT)_{12}$	174-243	5	0.321	0.731	0.6682	0.0000

Ta: annealing temperature; N_A: number of alleles; H_O: observed heterozygosity; H_E: expected heterozygosity; PIC: polymorphism information content; HWE: Hardy-Weinberg equilibrium.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

Research supported by the National High-Tech Research and Development Program of China (#2012AA10A405-3), and was funded by the National Natural Science Foundation of China (#31272658), the Special Fund of Agroscientific Research in the Public Interest (#201403008), the Natural Science Foundation of Hainan Province of China (#314086), and the Science and Technology Planning Project of Guangdong Province, China (#2014B030301064).

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