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RESEARCH ARTICLE

Characterization of polymorphic microsatellite markers and genetic diversity in the Hong Kong oyster *Crassostrea hongkongensis* using paired-end Illumina shotgun sequencing

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Abstract We isolated and characterized a total of 41 polymorphic microsatellite loci in *Crassostrea hongkongensis*, an oyster restricted to southern China. Loci were screened in 30 wild individuals from southern coastal region in China. The number of alleles per locus ranged from 2 to 18, observed heterozygosity and expected heterozygosity ranged from 0.000–0.867 to 0.066–0.926, respectively. To study the genetic diversity and population structure of this species form 3 locations, such as Xiamen, Zhuhai and Qinzhou, 12 loci were chosen from the 41 loci. The three sample sets displayed different diversity levels and observed heterozygosity ranged from 0 to 0.867. Significant genotype heterogeneity ($P < 0.001$) over most loci indicated that the samples are not drawn from the same gene pool. The pairwise F_{ST} values between XM and ZH, and QZ and ZH indicate that there were significant deviations in genetic differentiations for the Hongkong oyster populations from these locations. These new microsatellite loci will facilitate future studies of population structure and investigation of conservation genetics in this species.

Keywords *Crassostrea hongkongensis* · Hong Kong oyster · Microsatellite markers · Illumina sequencing · Genetic diversity

Introduction

The Hong Kong oyster (*Crassostrea hongkongensis*) is endemic and one of the most important economic and extensively cultured oyster species along the southern coastal areas of China. *C. hongkongensis* was known as *Crassostrea rivularis* previously (Li et al. 1988), and some researchers thought it was putative *Crassostrea gigas*. Until 2003, Lam and Morton verified that *C. hongkongensis* was a genetically distinct taxon by phylogenetic analyses based on the *cytochrome oxidases I* and *16S* data (Lam and Morton 2003). Hong Kong oyster is hitherto only found in three provinces of China (Fujian, Guangdong and Guangxi) and Hong Kong. It is a popular marketing product in southern of China and supporting one of the largest coastal aquaculture industries in this area. However, the wild Hong Kong oyster has declined in recent years owing to overexploitation and habitat destruction, and the farming of *C. hongkongensis* has been relying on the natural seeds until now. Such anthropogenic threats highlight the growing need for conservation of *C. hongkongensis*, and it becomes prerequisite to unravel the population dynamics essential for planning and adopting conservation measures.

Microsatellites are frequently used in studying the population genetic structure for the sustainable management of natural resources, due to these advantages of high reproducibility, codominant inheritance and genome wide abundance (Simko 2009). Today these markers can be developed quickly and at low cost for most species using next-generation sequencing (NGS) (Berman et al. 2014). At present, few microsatellites have been reported in *C. hongkongensis* (Xia et al. 2009; Li and Yu 2010; Xiao et al. 2011), but more polymorphic loci are definitely required for further work such as genetic mapping and trait improvement studies.

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Based on the cost effective method for microsatellite development provided by next-generation sequencing technology, we developed and characterized 41 novel microsatellite marks of *C. hongkongensis*. The study also presents the results on genetic variability and divergence in three populations of *C. hongkongensis* from different provinces in China to evaluate the potential of identified microsatellite loci in population structure of the important species.

Materials and methods

Total RNA was extracted from gill tissues of six individuals of *C. hongkongensis* by TRIzol reagent (Invitrogen) according to the manufacturer's instructions, respectively. We balanced pooled the RNA of six individuals and prepared cDNA samples following the protocol described in (Ng et al. 2005). An Illumina paired-end shotgun library was prepared by shearing to 300–500 bp by a UTR200 sonication device (Hielscher Ultrasonics GmbH) and following the standard protocol of the Illumina TruSeq DNA Library Kit. Sixty million reads were assembled by Trinity and analyzed with the perl script MIcroSAtellite (MISA; http://pgrc.ipk-gatersleben.de/misa/) for microsatellite mining. The parameters were designed for identification of perfect di-, tri-, tetra-, penta- and hexanucleotide motifs with minimum of repeat numbers of 6, 5, 5, 4 and 4, respectively. Based on MISA results, Primer 3 v2.23 (http://primer3.sourceforge.net) was used for primer design, with a total of 2,045 contigs found to possess optimal priming sites.

One hundred and fifty primer pairs were tested for amplification and polymorphism on DNA obtained from 30 individuals collected in Zhuhai (Guangdong Province), China. PCR amplifications were performed in 10 μL volumes containing 0.25 U *Taq* DNA polymerase 1× PCR buffer, 0.2 mM dNTP mix, 2.0 mM MgCl2, 1 μM of each primer and about 100 ng template DNA. The amplifications were programmed using following conditions: 94 °C for 3 min, followed by 35 cycles at 94 °C for 1 min, annealing temperature (Table 1) for 1 min, 72 °C for 1 min, then a final extension at 72 °C for 5 min. The amplification products were separated on 6 % denaturing polyacrylamide gels using a 10 bp ladder by silver staining. Forty-one microsatellite loci were deposited in GenBank (accession numbers KMO86772-KMO86812).

Twelve loci from these loci were characterized in 3 populations of: Xiamen (Fujian Province, 48 individuals), Zhuhai (Guangdong Province, 37 individuals), Qinzhou (Guangxi Province, 45 individuals). Allelic variation at the microsatellite loci were determined using MICROSATELLITE ANALYSER software (Dieringer and Schlötterer 2003) for the number of alleles, the observed heterozygosity (H_o) and expected heterozygosity (H_e). And it was also used to calculate global and population pair-wise $F_{ST.}$ Tests for linkage disequilibrium and Hardy–Weinberg equilibrium were performed using Genepop v4.0 (http://genepop.curtin.edu.cn/). Finally, all loci were assessed using MICRO-CHECKER to check for null alleles and scoring errors (Van Oosterhout et al. 2004).

Results and discussions

In this study, we amplified successfully 41 polymorphic microsatellite loci among 150 primer pairs developed. The number of alleles per locus range from 2 to 18 (Table 1), with a mean of 6.59 alleles per locus. The Ho and He values ranged from 0.000–0.867 to 0.066–0.926, respectively. Eight loci significantly deviated from Hardy–Weinberg equilibrium (HWE) after Bonferroni corrections. Tests for linkage disequilibrium revealed a nonrandom association ($P < 0.01$) between two pair of loci (Ch07–Ch08, Ch24–Ch29).

Of these 41 polymorphic microsatellite loci, 12 loci were further considered for population genetic analysis of *C. hongkongensis* in a total of 130 samples from 3 locations. *Ho* at all 12 polymorphic loci in the 3 populations ranged from 0 in CH35 of QZ to 0.867 in CH8 of ZH, whereas the range of H_e was 0.084 in CH40 of QZ to 0.840 in CH39 of ZH (Table 2). There was no significant association indicative of linkage disequilibrium between any pair of microsatellite loci for any population ($P < 0.05$), therefore assumed that allelic variation at microsatellite loci was considered independent.

The exact tests for fitness to HWE on all loci indicate that, there are four loci in QZ and six loci in XM show departures from HWE expectations (Table 2). The observed pattern of departures from HWE expectations may have been influenced by both the local presence of null alleles (Table 3) in moderate frequencies and by population processes (Kajtoch et al. 2012), (e.g. the larvae of the Hongkong oyster may have been inhabited by distinct populations due to their planktonic life). Moreover, the determination of inbreeding coefficient (F_{IS}) through partitioning of genetic variability as suggested Wright (1965) and Weir and Cockerham (1984) has been widely used to determine if the population has excess or deficit of heterozygote.

Genetic diversity is generally the result of long-term evolution and it represents the evolutionary potential of a species. To survive and adapt to an unstable environment, a species has to evolve and accumulate genetic variation (Lande et al. 1996; Oostermeijer et al. 2003). Tables 2 and 4 in this study clearly demonstrate the presence of fair levels of polymorphism in *C. hongkongensis*. The pairwise

Table 1 Characterization of 41 microsatellite loci isolated from *Crassostrea hongkongensis*

Locus	Primer sequence(5′–3′)	Repeat motif	No. of alleles	Size range (bp)	T_a (°C)	H_o	H_e	*P* value	GeneBank accession number
Ch01	F:TGTCTCCTTCCACCACTCCT	$(AC)_6$	2	190–194	60	0.167	0.413	0.0018	KMO86772
	R:CAAATGATGACGGCTCACTG								
Ch02	F:GGCCATCCCAAGATACAGTC	$(TA)_8$	5	222–230	60	0.3000	0.462	0.0092	KMO86773
	R:TCCTTGTAAACCCTCTCGGA								
Ch03	F:CAGACAAACACACAGCCCAT	$(GA)_9$	18	242–284	60	0.733	0.926	0.0605	KMO86774
	R:GTCACTGCGAGTCCCATCTT								
Ch04	F:TCGGAGGCATTGACAGAAAT	$(TG)_{10}$	3	278–286	60	0.067	0.128	0.1013	KMO86775
	R:TGCCATTGCTGTAGACTTGG								
Ch05	F:TAAGACTGGCAATCACGTCG	$(AC)_6$	3	204–214	60	0.333	0.288	1.0000	KMO86776
	R:ACGTCAGCAGGGATCTCAAT								
Ch06	F:GGCTTCATTAATGCCTCATAGA	$(AT)_6$	10	278–304	60	0.0000	0.827	1.0000	KMO86777
	R:CCTCGGTCTATCAGCTGTCC								
Ch07	F:ATTACTGGCAGATTCCACGC	$(AG)_6$	2	324–330	60	0.133	0.127	1.0000	KMO86778
	R:TGTGAGTTCCACACCAGGTT								
Ch08	F:TTTGTGGTATGTGGCTCCAA	$(AT)_6$	8	124–138	60	0.867	0.799	0.0418	KMO86779
	R:TACCCCATGAACTCAAATGG								
Ch09	F:GATGTGATTGCGGACGATAA	$(ATG)_5$	5	175–187	60	0.367	0.420	0.2103	KMO86780
	R:TTTGCCCTTTGGAATCTCAG								
Ch10	F:CGAGTTTCCCTCTCCCACTT	$(TG)_6$	2	240–246	60	0.267	0.235	1.0000	KMO86781
	R:AACATCAAACTCGGCTGGAG								
Ch11	F:ATCGATCCAGTGTCGGGTAG	$(TCG)_5$	2	251–257	60	0.833	0.494	0.0002*	KMO86782
	R:TCCATCAATCTTCGAGAGGC								
Ch12	F:CCAAGGGCAAGGATAATTGA	$(TA)_6$	2	240–246	60	0.100	0.155	0.1637	KMO86783
	R:TGATGGTAGATCATGCGATGT								
Ch13	F:ATGGCCATTACAGCAAAGGA	$(CTC)_5$	3	448–460	60	0.567	0.430	0.1662	KMO86784
	R:TGGTGATGAGGAATCATTGG								
Ch14	F:GGATGCACCCTCAAACAACT	$(AG)_6$	2	223–227	60	0.167	0.155	1.0000	KMO86785
	R:CGGCTCGTGTTACAGTGGTA								
Ch15	F:CGATACATGCTTGCTTGCTT	$(AT)_7$	14	256–296	60	0.733	0.895	0.0016	KMO86786
	R:TGTTGACGACGTGCATGATA								
Ch16	F:CATGAACTTCCCTGTTCCTGT	$(AC)_8$	2	236–242	60	0.067	0.183	0.1637	KMO86787
	R:TGCTTTGTTCTATTGTCGGC								
Ch17	F:TGATGTTGAGAACACTGCTCG	$(AT)_6$	4	198–230	56	0.125	0.264	0.0106	KMO86788
	R:AAGAAACATGTATGCCCTCCA								
Ch18	F:TTGATGTGTGCATGGGAGTT	$(AG)_7$	3	202–212	60	0.133	0.129	0.1544	KMO86789
	R:GGAAACATGGCTATTTCTGCTT								
Ch19	F:TCGGTATATGTGAATGCTCTGC	$(GA)_6$	2	175–181	56	0.333	0.413	0.1802	KMO86790
	R:CAGCACAGTGACTAGCTGATGA								
Ch20	F:CATCATTGTCTTCCGTGCAA	$(GA)_{11}$	17	272–312	60	0.379	0.903	0.0000*	KMO86791
	R:CCCGAAATGCAGTCTGTGTA								
Ch21	F:TGTTTCACGACTCTCGTTCG	$(TGT)_6$	4	155–164	60	0.6550	0.6950	0.0205	KMO86792
	R:GCGGAAGACATGGAGATTGT								
Ch22	F:TGCAGCGTTGCCTGTAGATA	$(AG)_9$	2	210–214	60	0.100	0.155	0.1650	KMO86793
	R:CACCTTACCAAGCTCGTTGC								
Ch23	F:GCTGAATGCAAACCAATTCTT	$(TA)_7$	16	270–312	60	0.333	0.866	0.0000*	KMO86794
	R:TGGGCAAATTTAAGACGGAG								
Ch24	F:CCAAGCAATTTATTGCCAGTT	$(TC)_6$	2	161–165	60	0.321	0.363	0.6049	KMO86795
	R:CCAGTTGAGTTTGCTAGGGTG								

Table 1 continued

Locus	Primer sequence(5′–3′)	Repeat motif	No. of alleles	Size range (bp)	T_a (°C)	H_o	H_e	P value	GeneBank accession number
Ch25	F:CATTGGCACAATTGTTAGCG	$(AT)_8$	13	122–156	56	0.333	0.805	0.0000*	KMO86796
	R:TTGCAAGCATCACAAGCACT								
Ch26	F:ACAGGAATGACGAGGTGGAC	$(ACAG)_5$	4	167–179	60	0.500	0.592	0.1605	KMO86797
	R:CACAAACATCCGAGACCTCC								
Ch27	F:TCACGGTAATTCTGATGCCA	$(AAG)_5$	11	170–206	60	0.533	0.876	0.0000*	KMO86798
	R:TGAGTCTGTCCGCTATGCAC								
Ch28	F:TCTCGATCGTCTGAATGTGC	$(GTT)_5$	2	270–276	60	0.100	0.381	1.0000	KMO86799
	R:CCAGTGAAACCTCCGACAGT								
Ch29	F:TGCAATCACAACATGCAACA	$(TTA)_5$	2	257–260	60	0.067	0.282	0.0005*	KMO86800
	R:AATGTAAAGGCACACGCACA								
Ch30	F:GACAATAGCCAACCGAAAGC	$(AG)_6$	2	252–272	60	0.500	0.381	0.1437	KMO86801
	R:GCACGAGCCTGTAGATTGAA								
Ch31	F:TCTGCCCTGTCGGTTTCTAC	$(GGA)_5$	3	170–176	56	0.067	0.066	1.0000	KMO86802
	R:TCCCACATTTCCTCTTCGTC								
Ch32	F:TGGACCCAAAGTTTCACACA	$(CA)_7$	3	188–192	56	0.607	0.527	0.4578	KMO86803
	R:GACGGTGGAGAATGGTTGTT								
Ch33	F:TGTCAAGCTGAGGTCCTCCT	$(AG)_6$	7	204–218	56	0.483	0.709	0.0063	KMO86804
	R:AGCTCCTGACAACCCACAAT								
Ch34	F:TGGCAGATACAGTCGTCCAG	$(TC)_6$	2	331–345	60	0.100	0.345	0.0008*	KMO86805
	R:TATACCAACCCGCTGACCTC								
Ch35	F:TGAAATGGACTCCGAAGAGG	$(AT)_7$	2	228–232	60	0.100	0.259	0.0065	KMO86806
	R:TGCTTCATCTTCCACAATTCC								
Ch36	F:GGCCATTACAGGTGCTTGTT	$(GAG)_6$	3	281–287	60	0.367	0.310	0.6246	KMO86807
	R:CAGCTACGACGTGTCAAGGA								
Ch37	F:AACCTGGAATAAATGCAGCC	$(AT)_8$	2	266–272	60	0.033	0.210	0.0004*	KMO86808
	R:CAGCCATCACATGTCATCAA								
Ch38	F:ACAGGCTGCACAACATGAAG	$(CA)_6$	2	242–250	60	0.067	0.183	0.0134	KMO86809
	R:TGCAATGTTCTGTCTAGGGATG								
Ch39	F:ATTGCCGGTCACAGAGTAGG	$(CA)_8$	8	158–172	60	0.700	0.840	0.0131	KMO86810
	R:CCTTTCCCATGAAAGTCCAA								
Ch40	F:ATGAGCATTGCCAACATCAA	$(TA)_6$	3	254–264	60	0.200	0.239	0.4144	KMO86811
	R:GAAGTTGCAAGCAACACCAG								
Ch41	F:AGAAATGATGACACAGCCGTT	$(TC)_8$	4	256–268	60	0.367	0.399	0.0369	KMO86812
	R:CAAGGCCTGAACACCTTGAT								

T_a annealing temperature, H_o observed heterozygosity, H_e expected heterozygosity

* Significant deviations from Hardy–Weinberg equilibrium after sequential Bonferroni correction ($P < 0.05/41$)

value of the coefficient of genetic differentiation (F_{ST}) was estimated between populations. The maximum F_{ST} 0.2339 was shown between the ZH and QZ populations and the minimum 0.009 between the XM and QZ populations. As Table 4 showed, pair-wise F_{ST} statistics between ZH and QZ, ZH and XM were significant, suggesting that the two population pairs were significantly different from each other ($P < 0.001$).

Several evolutionary forces, such as mutations, random genetic drift, gene flow and natural selection, influence the variation pattern of genomes and populations (Gaut et al. 2000). Random genetic drift due to isolation and mutations may be the important factor contributing to genetic differentiation of ZH and other two populations. Although the larvae of oysters has days of planktonic life, it is difficult for them to disperse from Qinzhou, Guangxi Province to

Table 2 Summary of genetic variation and heterozygosity statistics of twelve microsatellite loci in *C. hongkongensis*

Locus	Repeat motif	Population	No. of alleles	Size range (bp)	H_o	H_e	P	F_{IS}
CH05	$(AC)_6$	ZH	3	204–214	0.333	0.288	1.0000	−0.1623
		QZ	3	204–214	0.130	0.163	0.0201	0.2035
		XM	2	204–214	0.292	0.252	0.5707	−0.1605
CH08	$(AT)_6$	ZH	8	124–138	0.867	0.799	0.0418	−0.0857
		QZ	7	124–138	0.556	0.652	0.0008*	0.1228
		XM	6	124–138	0.660	0.664	0.0170	0.0063
CH10	$(TG)_6$	ZH	2	240–246	0.267	0.235	1.0000	−0.1373
		QZ	3	230–246	0.244	0.255	0.0179	0.0435
		XM	3	230–246	0.313	0.304	0.0346	−0.0292
CH13	$(CTC)_5$	ZH	3	448–460	0.567	0.430	0.1662	−0.3253
		QZ	2	454–460	0.174	0.161	1.0000	−0.0843
		XM	3	448–460	0.646	0.466	0.0045	−0.3929
CH21	$(TGT)_6$	ZH	4	155–164	0.655	0.695	0.0205	0.0584
		QZ	2	158–164	0.348	0.444	0.1807	0.2191
		XM	4	155–167	0.396	0.509	0.0049	0.2235
CH22	$(AG)_9$	ZH	2	210–214	0.100	0.155	0.1650	0.3603
			2	210–214	0.283	0.494	0.0058	0.4304
			3	210–220	0.265	0.523	0.0002*	0.4956
CH26	$(ACAG)_5$	QZ	4	167–179	0.500	0.592	0.1605	0.1572
		XM	4	167–179	0.478	0.531	0.7702	0.0996
			4	167–179	0.265	0.537	0.0000*	0.5085
CH30	$(AG)_6$	ZH	2	252–272	0.500	0.381	0.1437	−0.3182
		QZ	2	252–272	0.61	0.291	0.6011	0.1030
		XM	2	252–272	0.298	0.384	0.1371	0.2269
CH35	$(AT)_7$	ZH	2	228–232	0.100	0.259	0.0065	0.6184
		QZ	2	228–232	0.000	0.296	0.0000*	1.0000
		XM	2	228–232	0.104	0.237	0.0017*	0.5624
CH39	$(CA)_8$	ZH	8	158–172	0.700	0.840	0.0131	0.1686
		QZ	4	158–172	0.289	0.621	0.0000*	0.5376
		XM	3	164–172	0.312	0.529	0.0000*	0.4115
CH40	$(TA)_6$	ZH	3	254–264	0.200	0.239	0.4144	0.1655
		QZ	2	260–264	0.087	0.084	1.0000	-0.0345
		XM	4	252–264	0.081	0.389	0.0000*	0.7916
CH41	$(TC)_8$	ZH	4	256–268	0.367	0.399	0.0369	0.0833
		QZ	4	256–268	0.348	0.553	0.0000*	0.3731
		XM	4204	256–268	0.367	0.502	0.0000*	0.2700

H_o observed heterozygosity, H_e expected heterozygosity, F_{IS} inbreeding coefficient

ZH, oyster population from Zhuhai, Guangdong Province; QZ, oyster population from Qinzhou, Guangxi Province; XM, oyster population from Xiamen, Fujian Province

* Significant deviations from Hardy–Weinberg equilibrium after sequential Bonferroni correction ($P < 0.05/41$)

Xiamen, Fujian Province. *C. angulate* is dominant species in Fujian Province and *C. hongkongensis* mainly distributes around the estuary of Jiulong River (Zeng and Ning 2011). Most of the seeds of the Hongkong oyster were from Qinzhou (Su 2006). It is apparent that human factor may be the reason for the result that there is no significantly genetic differentiation.

In conclusion, the present study is the first attempt at the illustration of genetic structure of the Hongkong oyster in Southern China. The effect of human factor is one of the main factor to disturb the genetic differentiation of populations in different locations from this study. These 41 novel polymorphic microsatellites will facilitate the study of the genetic structure of *C. hongkongensis*, and provide

Table 3 Summary statistics of null allele frequencies in *C. hongkongensis*

Locus	Null allele frequency		
	ZH	QZ	XM
CH04	0.0000	0.1008	0.0000
CH08	0.0245	0.2252	0.0474
CH10	0.0000	0.1019	0.0902
CH13	0.6708	0.9089	0.6144
CH21	0.1003	0.3901	0.1180
CH22	0.9309	0.6594	0.1833
CH26	0.0996	0.0627	0.2336
CH30	0.0000	0.2085	0.3262
CH35	0.8944	0.9068	0.9014
CH39	0.0891	0.4648	0.3631
CH40	0.1729	0.0000	0.3939
CH41	0.1231	0.2265	0.2017

Table 4 Pairwise F_{ST} between three natural populations of *C. hongkongensis* studied through twelve polymorphic microsatellite loci

Population	QZ	ZH
XM	0.0090	0.2037*
ZH	0.2339*	–

* Significant after sequential Bonferroni adjustment ($P < 0.001$)

an essential component for formulating meaningful conservation strategies for this species.

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Conflict of interest The authors declare that they have no conflict of interests.

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