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Short communication

The genetic diversity of wild and cultivated Manila clam (*Ruditapes philippinarum*) revealed by 29 novel microsatellite markers

Liwen Jiang, Hongtao Nie *[,1], Chen Li, Dongdong Li, Zhongming Huo, Xiwu Yan *

College of Fisheries and Life Science, Engineering and Technology Research Center of Shellfish Breeding in Liaoning Province, Dalian Ocean University, Dalian, 116023, China

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ABSTRACT

Background: Microsatellite loci often used as a genetic tool for estimating genetic diversity population variation in a wide variety of different species. The application of microsatellite markers in genetics and breeding includes investigating the genetic differentiation of wild and cultured populations, assessing and determining the genetic relationship of different populations. The aim of this work is to develop several microsatellite markers via high-throughput sequencing and characterize these markers in commercially important bivalve *Ruditapes philippinarum*.
Results: Among the two populations of *R. philippinarum* studied, 110 alleles were detected. The number of alleles at the cultured population ranged from 3 to 17 (mean NA = 6.897) and wild population ranged from 2 to 15 (mean NA = 6.793). The observed and expected heterozygosities of cultured population ranged from 0.182 to 0.964, and from 0.286 to 0.900, with an average of 0.647 and 0.692, respectively. The observed and expected heterozygosities of wild population ranged from 0.138 to 1.000, and from 0.439 to 0.906, with an average of 0.674 and 0.693, respectively. The polymorphism information content ranged from 0.341 to 0.910 with an average of 0.687. Sixteen and thirteen microsatellite loci deviated significantly from Hardy–Weinberg equilibrium after correction for multiple tests in cultured and wild population, respectively.
Conclusions: Twenty-nine novel microsatellite loci were developed using Illumina paired-end shotgun sequencing and characterized in two population of *R. philippinarum*.
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1. Introduction

The Manila clam, *Ruditapes philippinarum*, is an economically important bivalve species of the China aquaculture industry and is widely distributed across the coasts of China. The world production of this species was about 4.0 million metric tons in 2014 [1]. As one of the commercially important resources for the shellfish fisheries in China, development of *R. philippinarum* breeding and aquaculture has drawn a considerable attention among the farmers [2]. In recent years, the scale of artificial breeding of *R. philippinarum* has been developing rapidly, and the breeding area has been expanding, which is mixed with wild populations. This human activity has affected the genetic diversity structure of *R. philippinarum*. In addition, during successive selection process, no genetic material was introduced to the cultured population, which might reduce the genetic diversity of the closed populations by selective pressure and inbreeding [3,4]. Therefore, the analyses of genetic status of the *R. philippinarum* populations are necessary to maintain the genetic diversity of the valuable resources.

Microsatellites or Simple sequence repeat (SSR) has many advantages compared with other DNA markers such as high polymorphism, good repeatability, and especially in the different population has a strong commonality [5,6]. It is widely used in molecular genetic research including parentage determination [7], population structure analysis [8] and genetic linkage mapping [9]. In addition, SSR is considered as one of the best molecular markers for genetic diversity analysis and population genetics study [10,11]. It is revealed that genetic diversity is related to the sustainability of populations [12,13]. Therefore, it is essential to investigate the impact of artificial selection on genetic diversity of artificially cultivated populations for further aquaculture production. So far, a number of studies on the genetic diversity have been conducted in several economically important shellfish species, such as *Crassostrea gigas* [14], *Meretrix petechialis* [15], and *R. philippinarum* [16,17,18]. Although some microsatellite loci are available in *R. philippinarum* [19,

* Corresponding authors.
E-mail addresses: htnie@dlou.edu.cn (H. Nie), yanxiwu@dlou.edu.cn (X. Yan).
[1] Present address: Department of Anatomy, Physiology, and Pharmacology, College of Veterinary Medicine, Auburn University, Auburn, Alabama 36849-5519, USA.
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20,21,22,23], more polymorphic microsatellites are still required in this species to obtain a better understanding of the clam genetics.

With the continuous development of sequencing technology, the genome sequencing of the number of shellfish species has been completed including *R. philippinarum* [24]. High-throughput sequencing offers significant advantages in terms of technical simplicity, cost, and allow for fast and efficient detection of microsatellite markers [25,26]. Illumina paired-end shotgun sequencing was used to develop and characterize microsatellite loci for some bivalve species [27]. The purpose of the present study was to use Illumina paired-end shotgun sequencing to develop and characterize microsatellite loci for Manila clam. Meanwhile, these markers were utilized to analyze the genetic diversity in cultivated and wild populations of *R. philippinarum*. These new microsatellite markers will facilitate future genetic linkage mapping and population studies on the genetic diversity and structure of *R. philippinarum*.

2. Materials and methods

R. philippinarum are collected from wild population of Changxingdao (CX) (39° 22′N,121° 15′E) and cultivated population of Zhuanghe (ZH) (39°43′N,123°01′E). The Manila clam is not an endangered or protected species, so no specific permits were required for the study. The wild population was obtained by artificial digging from clam natural distribution area. Genomic DNA of each specimen was extracted from adductor muscle tissue by Marine Shellfish Extraction Kit (TIANGEN) DNA and stored in −20°C. Using the Covaris ultrasonic processor (Covaris, USA), DNA samples were randomly sheared to ~230 bp in size. Fragmented DNA was endrepaired using T_4 DNA polymerase and an 'A' base was added to the ends of double strand break DNA. Next, DNA adaptors (Illumina, USA) with a single 'T' base overhang at the 3′ end were ligated to the above products. These products were then separated on an agarose gel, excised from the gel, and purified. The adaptor modified DNA fragments were enriched via PCR amplification using Illumina paired-end PCR primers (Illumina, USA). The concentration of the libraries was initially measured by Qubit®2.0 (Life technologies, USA). The libraries were diluted to 1 ng/μl and the Agilent Bioanalyzer 2100 (Agilent, USA) was used to test the insert size of the libraries. The libraries were sequenced on the Illumina HiSeq 2500 platform (Illumina, USA) by Novogene Bioinformatics Institute, Beijing, China. Paired end (PE) reads with 125 bp were determined and the clean reads were collected from sequenced reads, which were pre-processed to remove adaptors and low quality paired reads. The following criteria were used to remove the low quality reads: i) containing more than 10% 'N's; ii) more than 50% bases having low quality value (Phred score ≤ 5), and iii) containing adaptor reads. The primer-pair design process was automated to submit large batches of sequences to a local installation of the program PRIMER3 (version 2.0.0), and was implemented in the Perl program PAL_FINDER_v0.02.03.

During the designing of locus specific primers, a random selection of penta-and hex-nucleotide microsatellites were used, in order to simplify the process of scoring during genotyping. Twenty-nine primer pairs were tested on 30 cultured individuals from ZH, and 30 wild individuals from CX, respectively. Polymerase Chain Reaction (PCR) amplifications were performed in a 10 μl reaction volume containing 0.5 U easy Taq DNA polymerase (Takara, Japan), 1× PCR buffer, 0.2 mM dNTP, 0.4 μM of each primer set, and about 25 ng template DNA. PCRs were performed using a PCR thermal cycler as follow: 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 45 s at optimal annealing temperature, and 72°C for 30 s; then 72°C for 5 min. Amplification products were resolved on an 8% polyacrylamide gel and visualized by silver staining. Allele sizes were determined by using a 10 bp DNA ladder (Invitrogen).

A total of 96,478 of the resulting reads were analyzed using PAL_FINDER_v0.02.03. Reads containing di-, tri-, tetra-, penta-, and hexanucleotide microsatellites were identified. PRIMER3 (version 2.0.0) was used to identify primer regions based on the reads containing putative microsatellite regions. Microsatellites formed by penta-and hexa-nucleotide motifs were selected for primer design, in order to simplify the process of scoring during genotyping. A total of 150 microsatellite primers were designed using PRIMER 5.0 program (http://www.premierbiosoft.com/). For the successful primers, estimated fragment length, the number of alleles (NA), observed (Ho) and expected (He) heterozygosities were using the program MICROSATELLITE ANALYSER (MSA) [28]. Deviations from Hardy–Weinberg equilibrium (HWE) and Linkage Disequilibrium were performed by GENEPOP 4.0 [29]. Polymorphism information contents (PICs) were performed by PIC_CALC 0.6. Sequential Bonferroni corrections [30] were applied for all multiple tests. The MICRO-CHECKER 2.2.3 software [31] was used to check microsatellites for null alleles and scoring errors.

3. Results and discussion

Twenty-nine of 150 screened primers (19.3%) were found to be polymorphic among 8 individuals of *R. philippinarum*. There are successfully amplified from the 60 *R. philippinarum* individuals (Table 1). In total, 110 alleles were detected at the two microsatellite loci analyzed. Rpg14043 with 22 alleles was the most polymorphic microsatellite, while Rpg7789 was the least variable (Table 1). The PIC ranged from 0.341 to 0.910 with an average of 0.687. According to Botstein et al. [32], the PIC value higher than 0.5 were highly polymorphic, ranged from 0.25 to 0.5 were moderate polymorphism. In this study, twenty-eight microsatellite loci were highly polymorphic, while only one locus Rpg7789 showed the moderate polymorphism (Table 1). These microsatellite loci will be useful for further studies on the population structure and genetic variation of this species.

All 29 microsatellite loci were polymorphic in two populations of *R. philippinarum* and the levels of polymorphism varied among loci. The genotype data of the *R. philippinarum* from cultivated and wild populations were used to calculate the parameters of N_A, H_O, H_E, and PIC for assessing the genetic diversity level (Table 2). Estimated fragment size at each locus was between 100 and 200 bp (base-pairs). The number of alleles (N_A) at the ZH farm population ranged from 3 to 17 (mean $N_A = 6.897$) and CX wild population ranged from 2 to 15 (mean $N_A = 6.793$). At the population level, the observed and expected heterozygosities of ZH population ranged from 0.182 to 0.964, and from 0.286 to 0.900, with an average of 0.647 and 0.692, respectively. The observed and expected heterozygosities of CX population ranged from 0.138 to 1.000, and from 0.439 to 0.906, with an average of 0.674 and 0.693, respectively.

In this study, 16 loci in ZH population deviated significantly from Hardy–Weinberg equilibrium (HWE) and 13 loci in CX population deviated significantly from HWE after correction for multiple tests (Table 2), which may be due to the presence of null alleles and sampling effect. The MICRO-CHECKER analysis suggested that there were no indications for scoring error due to stuttering or for large allele dropout. Ten null alleles were detected in ZH population and eight null alleles were detected in CX population. Five loci (Rpg 10,677, Rpg10579, Rpg7789, Rpg10939 and Rpg14043) have null alleles in both two populations. Null alleles are probably a major cause for the heterozygote deficiency observed from SSR analysis of populations [14]. Fourteen pairs of loci were in linkage disequilibrium in ZH population and two pairs of loci were in linkage disequilibrium in CX population after Bonferroni corrections ($P < 0.01$).

According to previous reports, successive closed breeding with a limited number of parental founders could lead to a reduction in genetic diversity and the effective population size, which could increase the rate of both inbreeding and genetic drift [33]. In the present study, the average of expected heterozygosities (0.692 and

Table 1
Characterization of 29 polymorphic microsatellite markers developed from *R. philippinarum*.

Locus	Accession number	Repeat motif	Primer sequence (5′–3′)	T_a (°C)	N_a	H_o	H_E	PIC	Size range (bp)
Rpg7264	KX267896	$(TATGA)_4$	F:TGATTGAGAAAATTGAAATAGAGCTACA R: CGTTACAAAACTCGTCAATGATGT	60	10	0.903	0.804	0.836	108–144
Rpg7789	KX267897	$(TCTGT)_4$	F:TGACGAGGAACTGTTATGTTCAAGTC R:TCCAAGCCGCTCTATCATCTTCTA	63	3	0.431	0.404	0.341	128–136
Rpg7797	KX267898	$(AATTA)_4$	F:TTGGTTTCTATGCAAATAAGCAATG R:AAAAGATCAGGATGAGGATCAACTG	61	8	0.184	0.468	0.641	120–164
Rpg8392	KX267899	$(TTTTA)_4$	F:AACGTTATATGTTGCCGTGTTGG R:CATTTTGTTATTGTCAAGACACAGTTGA	63	4	0.931	0.639	0.655	104–116
Rpg8969	KX267900	$(AGTAC)_4$	F:CTTTTAAAAATTCAGGTGAGACCG R:GCCTTTAAAGTCACATGATTGGA	60	8	0.882	0.820	0.778	112–140
Rpg9635	KX267901	$(TATAT)_4$	F:AAAGTTCAGCCAAGTTGGAGACAT R:AACTGACACTGGCCTCAATACAAA	62	18	0.655	0.786	0.792	156–200
Rpg9694	KX267902	$(ATGTT)_4$	F:CATAATTCACATTTTCCCATTTGC R:GCATAAGTTTTACATGACATGACATCAC	61	12	0.964	0.753	0.738	100–132
Rpg10331	KX267905	$(GCAAT)_4$	F:AAAAGAAGATTTCCTTCAGCCTTG R:GGACTTTCCCGACTTTAAAACAGA	61	11	0.909	0.781	0.754	108–136
Rpg10334	KX267906	$(TATTA)_4$	F:AATTGTGAGATGCCTCAAGAAAGG R:TATTTATTTTTCGGCAGTCCAAGC	62	7	0.457	0.589	0.692	116–136
Rpg10418	KX267907	$(ATCAT)_5$	F:TGAACGACTTGAGTTTTTGGACTTC R:TGGAAGTATCTCTTTTCTTCACGCA	63	5	0.609	0.642	0.563	140–165
Rpg10419	KX267908	$(TGTTC)_4$	F:GTAAAATCCTCCATGCCATACCAA R:ATTTCGACCCTTTCCGTCAATC	63	9	0.536	0.785	0.817	144–180
Rpg10440	KX267909	$(ATTAC)_5$	F:GGTTGCTGTTCAAAAAGCATGAC R:TGCACTTTTGTAAAGAATGAGGCA	63	7	0.830	0.634	0.522	105–120
Rpg10579	KX267910	$(ATACT)_4$	F:GTATTGGTGATTGCTAAACCTCGG R:ATCGTCGAGAAAGTGTACACGACA	63	9	0.251	0.6	0.544	116–136
Rpg10677	KX267911	$(TGTAT)_5$	F:AACTGTAGAAATAGCCGTTCTGCG R:TATTGTGATGATCGGAACTGAACG	63	5	0.327	0.613	0.544	120–140
Rpg10939	KX267912	$(TCATG)_4$	F:CTTCCCCTGGGACTGAAAAATTA R:TCTTTAAGCATTGCCCTCTCTAGTTTA	62	8	0.320	0.517	0.536	136–180
Rpg11008	KX267913	$(AAATT)_4$	F:CAAAGCCATTGCACAAACAGATAA R:GGTCGATTTTCGTGAAACATCATT	63	8	0.779	0.696	0.727	120–152
Rpg11148	KX267914	$(AAAACA)_4$	F:TGTATTCATTCTTTGGCTTTTAGGC R:ATTGCATTTTGCGGAGGTAATAAA	61	12	0.959	0.783	0.751	104–128
Rpg11931	KX267915	$(TGGTA)_4$	F:ATTGTATGGCATGGAATAGCACG R:CCATGCCACATACCACATACCATA	63	14	0.938	0.818	0.836	124–164
Rpg12144	KX267916	$(TTATG)_4$	F:TGCTCATGCTATTTTATGCCAAAT R:CGTAAATTTCACCGTTAAACCACTG	61	5	0.644	0.686	0.636	104–120
Rpg12236	KX267917	$(ATATG)_4$	F:GCTATATGATGCGATGTGATATGATG R:GCGACATATTAAGCACGCGA	61	17	0.966	0.850	0.862	128–172
Rpg12135	KX267918	$(ATAAC)_4$	F:GAGGCATGCAGCTTTAATCAAAAT R:TTCCATCGAAATCTAAACCGAAAG	62	9	0.636	0.714	0.752	140–164
Rpg12623	KX267919	$(ATGTT)_5$	F:AAGCGTATACATGATTTTGTGTTTTGA R:ACAGTGACCCTACCTTTCACCTTG	61	9	0.572	0.7	0.676	125–150
Rpg12921	KX267920	$(ATGTT)_4$	F:TTCTGACTAGGTGAATGTAACTGTGC R:ACATGTAAGCATGGAAATACAAAATG	60	10	0.556	0.587	0.568	116–136
Rpg13399	KX267921	$(TTCTA)_4$	F:GATAAGCCTGAAAAGGCCGATAAT R:TTTGCACAGAGAGAAAAACACGAG	62	12	0.795	0.788	0.781	108–140
Rpg13518	KX267922	$(TGTTC)_5$	F:TGTTGTATATATGTGTTCATTGCATGTT R:AAATCTAAACTTGTTGATGTAGTTGGC	60	4	0.764	0.599	0.516	105–120
Rpg14043	KX267923	$(TACCA)_4$	F:CGTTCCGTACCGTATCGTATCC R:ATTGAACATGCTGTTGGCATAGTG	62	22	0.5	0.903	0.910	100–200
Rpg14340	KX267924	$(TTTTG)_4$	F:GACCTCACAAATCAAGTTCTGTTTGA R:AAGGATTCAACCACACATTCGGTA	62	9	0.946	0.747	0.759	120–156
Rpg14764	KX267925	$(AGAAC)_5$	F:GGCTCGCCAGTTGTCTAGTATTGT R:GCTCTGTGTAGCTCATTGTCAGGA	62	6	0.639	0.643	0.645	110–140
Rpg12489	KX267926	$(AAATC)_4$	F:TAGTGTCTGCTGAGGTAAGGACCC R:CAGATTTTGAATCATAACCGAGGC	63	6	0.532	0.746	0.739	104–140

T_a: annealing temperature of each primer pair, N_a observed number of alleles, H_O observed heterozygosity, H_E expected heterozygosity PIC polymorphism information content.

0.693) and the average number of alleles (6.897 and 6.793) in populations ZH and CX, respectively, were both at a similar high level, indicating that the genetic diversity of the closed breeding populations was not seriously affected by mass selection. However, the farmed population ZH have more loci deviated significantly from HWE after correction for multiple tests than the wild population CX. Further studies with more samples are needed for genetic monitoring in fine scale populations. So far, with the continuous expansion of the scale of aquaculture, the resources of the wild clam decreased gradually, may lead the decline of resources and genetic diversity. Therefore, we suggested to reinforce the protection and conservation of this species to ensure the sustainable utilization of clam resources. Information on the genetic variation and differentiation in population genetics is useful for future genetic improvement by selective breeding and design suitable management guidelines for *R. philippinarum*.

Conflict of interest

The authors declare no conflict of interest.

Table 2
Analysis of genetic diversity in wild and cultivated Manila clam *R. philippinarum*.

Locus	ZH					CX				
	N_A	H_O	H_E	PIC	*P*	N_A	H_O	H_E	PIC	*P*
Rpg7264	8	0.880	0.825	0.783	0.1687	8	0.926	0.783	0.743	0.0033
Rpg7789	3	0.207	0.372	0.326	0.0101	2	0.138	0.436	0.336	0.0009
Rpg7797	4	0.211	0.286	0.261	0.3882	6	0.158	0.650	0.567	0.0000*
Rpg8392	4	0.952	0.669	0.587	0.0000*	4	0.909	0.610	0.529	0.0031
Rpg8969	8	0.880	0.827	0.784	0.0000*	7	0.885	0.812	0.767	0.0000*
Rpg9635	11	0.552	0.759	0.710	0.0000*	15	0.759	0.812	0.787	0.1703
Rpg9694	9	0.964	0.744	0.689	0.0000*	10	0.963	0.762	0.717	0.0000*
Rpg10331	7	0.852	0.767	0.714	0.0000*	8	0.967	0.795	0.749	0.0000*
Rpg10334	5	0.650	0.603	0.548	0.4897	4	0.263	0.575	0.488	0.0024
Rpg10418	4	0.679	0.662	0.579	0.0042	5	0.538	0.623	0.552	0.0078
Rpg10419	5	0.571	0.761	0.706	0.0000*	7	0.500	0.809	0.758	0.0000*
Rpg10440	6	0.846	0.689	0.603	0.0709	4	0.815	0.579	0.474	0.0000*
Rpg10579	7	0.310	0.596	0.526	0.0043	7	0.192	0.603	0.553	0.0000*
Rpg10677	4	0.321	0.583	0.514	0.0004	5	0.333	0.643	0.572	0.0000*
Rpg10939	3	0.182	0.317	0.282	0.0064	7	0.458	0.716	0.675	0.0046
Rpg11008	8	0.800	0.840	0.804	0.0000*	4	0.759	0.552	0.455	0.0012
Rpg11148	10	0.952	0.787	0.737	0.0002*	6	0.966	0.778	0.723	0.0000*
Rpg11931	13	0.926	0.827	0.794	0.0056	7	0.950	0.808	0.760	0.1746
Rpg12144	4	0.679	0.841	0.575	0.0011	5	0.593	0.587	0.649	0.0000*
Rpg12236	15	0.588	0.649	0.828	0.1033	10	0.700	0.723	0.808	0.0014
Rpg12135	7	0.931	0.860	0.803	0.0000*	5	1.000	0.841	0.492	0.0030
Rpg12623	5	0.423	0.753	0.700	0.0000*	9	0.720	0.647	0.582	0.1011
Rpg12921	8	0.393	0.568	0.534	0.0002*	6	0.720	0.606	0.580	0.4186
Rpg13399	6	0.750	0.712	0.648	0.0300	11	0.840	0.844	0.806	0.0000*
Rpg13518	4	0.920	0.598	0.500	0.0000*	4	0.607	0.599	0.522	0.0008
Rpg14043	17	0.400	0.900	0.873	0.0000*	14	0.600	0.906	0.881	0.0000*
Rpg14340	5	0.960	0.718	0.658	0.0000*	9	0.931	0.776	0.728	0.0000*
Rpg14764	5	0.692	0.758	0.698	0.0000*	4	0.586	0.528	0.457	0.0653
Rpg12489	5	0.286	0.794	0.726	0.0000*	4	0.778	0.699	0.611	0.0670
Mean	6.897	0.647	0.692	0.653		6.793	0.674	0.693	0.679	

N_A: number of alleles, H_O: observed heterozygosity, H_E: expected heterozygosity. *Indicates significant departure from Hardy–Weinberg equilibrium after sequential Bonferroni correction ($P < 0.01$).

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