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Short communication

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

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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,

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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